The role of auxin in habituation of Lactuca sativa callus tissue

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THE ROLE OF AUXIN IN HABITUATION OF LACTUCA SATIVA CALLUS TISSUE

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Summary
IAA metabolism was compared in habituated and non-habituated Lactuca callus to determine its importance in the maintenance of the auxin-habituated state. IAA levels were estimated by HPLC-F and GC-MS(SIM) and the pathway and rate of IAA biosynthesis was studied in both tissues using ^{14}C tryptophan feeding and TLC autoradiography. An estimation of conjugated IAA levels was also attempted. Finally, auxin antagonists were used to determine the effect of reduced binding of endogenous IAA on cell proliferation.
Habituated and non-habituated callus was isolated from L. sativa leaf discs. These lines differed in colour, texture, growth rate, expression of organogenesis and auxin-requirement. NAA was an absolute requirement for the induction of callus from this tissue and habituated tissues arose readily from these calli.
Endogenous IAA levels fluctuated dramatically during a 21 day culture period in both tissues, but overall levels were higher in the habituated tissue at 3 stages during this period. Derivatisation of the "IAA" quantified by HPLC-F indicated that contamination was present, which was confirmed by the GC-MS(SIM) estimates.
IAA biosynthesis was not detected in either tissue, but uptake of tryptophan from the media was quantitative suggesting that tryptophan was metabolised in other anabolic pathways.
Endogenous IAA levels were significantly reduced (50 %) in non-habituated tissues by a growth-limiting period of culture in darkness. Attempts to determine whether this reduction was a result of conjugation of the IAA were unsuccessful due to contamination in the hydrolysed extracts.
DNA synthesis in habituated tissues was reduced by culturing with the auxin antagonist, 1-naphthoxyacetic acid. A reduction in uptake of the nucleoside at the plasmalemma was also detected.
The findings of this work support the hypothesis that endogenous IAA levels are important in the regulation of auxin-autonomous growth and cell proliferation in tissue culture. However, further extensive work is required before the regulatory mechanisms of IAA metabolism and the role of IAA in habituation can be established.

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Abbreviations

1-NAA	α-naphthylacetic acid
1-NOA	α-naphthoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2-NAA	β-naphthylacetic acid
4-Cl-IAA	4-chloroindole-3-acetic acid
5-OH-IAA	5-hydroxyindole-3-acetic acid
BUdR	5-bromodeoxyuridine
Ca^{2+}	calcium ion
cAMP	cyclic adenine monophosphate
DNA	deoxyribonucleic acid
ECD	electron capture detector
G1/S	transition stage in the cell cycle from the resting G1 phase to the S phase of DNA synthesis
G2/M	transition stage in the cell cycle from the resting G2 phase to the mitotic phase
GC	gas chromatography
GC-MS	combined gas chromatography mass spectrometry
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IAAsp	indole-3-acetyl aspartic acid
IAAGlu	indole-3-acetyl glutamic acid
IAGluc	1-0-indole-3-acetyl-β-D-glucose
IAInos	indole-3-myoinositol
IAld	indole-3-aldehyde

ICA	indole-3-carboxylic acid
IEt	indole-3-ethanol
IM	indole-3-methanol
IPyA	indole-3-pyruvic acid
k	kinetin
MS	mass spectrometry
5-MT	5-methyl tryptophan
NPD	nitrogen and phosphorous detector
ODS	octadecyl silicone
OxIAA	oxindole-3-acetic acid
PAA	phenylacetic acid
PCIB	chlorophenoxyisobutyric acid
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
mRNA	messenger RNA
T-DNA	portion of the Ti plasmid of Agrobacterium tumefaciens responsible for tumour induction
Ti plasmid	the infective plasmid of Agrobacterium tumefaciens
TLC	thin layer chromatography
trp	tryptophan
U.V.	ultra violet radiation

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Chapter 1

1.1 General introduction

Disorganised plant tumours cultured *in vitro* demonstrate an aberrant form of plant growth regulation. The existence of such neoplastic growths allows the mode of action of the key growth regulators in plant cell growth and division, auxin and cytokinin, to be investigated in tissues capable of proliferating in the absence of additional growth regulators.

There are two main sources from which plant tumours naturally arise. Firstly, plant cells may be transformed by bacterial or viral genetic information resulting in tumourous conditions. Crown gall, a disease affecting mainly dicotyledonous species (De Cleene and De Ley 1976) is one such condition. This disease is caused by the covalent integration of the Ti plasmid of *Agrobacterium tumefaciens* into the host plant cell genome (Bevan and Chilton 1982). The introduction of the double stranded RNA of the virus *Aureogenus magnivena* Black (Black 1945) is another example of this form of tumour induction.

Secondly, specific genetic crosses within susceptible plant species can give rise to tumour prone hybrids. The genetic tumours of certain *Nicotiana* hybrids are perhaps the most widely studied (Smith 1972).

There is also evidence that plant tumours may be induced *in vitro* by chemicals such as morphactins and aminofluorens when added to the growth medium of cultured plant tissues (Bednar and Linsmair-Bednar 1971). One

further form of growth regulator autonomy in disorganised plant tissue cultures often occurs spontaneously *in vitro*, where certain tissues express stable alterations in their nutritional requirements during culture. This phenomenon is termed habituation and was first identified by Gautheret (1955).

Habituation is usually observed as a loss of requirement for one or both of the growth regulators, auxin and cytokinin, routinely required for the initiation and maintenance of cell growth and division in plant tissue cultures. Cytokinin-habituation frequently exists in cultures which are still dependent on an exogenous supply of auxin for growth (eg. Meins and Binns 1978, Vyskot and Novak 1977, Kaminek and Lustinec 1974, Gamburg 1982), whilst habituation for auxin is usually manifested as autonomy for both auxin and cytokinin.

Other forms of habituation exist such as the loss of requirement for certain vitamins in the culture medium. Gautheret (1950) reported autotrophy for pantothenic acid in a culture of willow and total vitamin-autotrophy has been identified in tobacco cell cultures by Yoshida et al (1973). Habituation for growth regulators is, however, the most commonly observed condition and has been reported in a wide range of species, both mono- and dicotyledonous. These include *Lilium* (Sheridan 1968), maize (Hawes et al 1985), sugarbeet (Kevers et al 1981), *Arabidopsis* (Ondréj et al 1984), vine (Czosnowski 1952) and tobacco (Köves and Szabo 1987, Wyndaele et al 1985, 1988).

Despite the widespread occurrence of habituation, the underlying causes of the phenotype alterations involved remain unclear. The frequent observation of this condition in tissue culture does, however, illustrate the existence of, as yet, unidentified stresses which are imposed on plant tissues grown in the artificial *in vitro* culture regime.

Despite differences in the initiating agents of auxin and cytokinin-autonomous growth in plant cells, Braun (1972, 1978) has argued that fundamental cellular mechanisms exist in the establishment of this condition.

Evidence for the existence of auxin and cytokinin genes in crown gall-infected cells

Crown gall tissues are the most comprehensively studied neoplastic plant growths. These growths can be removed from infected plants and cultured *in vitro* after the removal of the infective bacteria by methods such as heat treatment and the use of antibiotics (Butcher 1977). Fully transformed crown galls are auxin and cytokinin-autonomous. Crown gall tissues offer great potential for examining the causes underlying growth regulator-autonomy in cultured plant tissues due to the knowledge that the Ti plasmid of the causative bacterium, *Agrobacterium tumefaciens*, encodes a number of gene products involved in tumour induction. These include genes directly involved in the biosynthesis of auxin and cytokinin (Nester et al 1984).

Morphogenetic crown gall mutants arising from transposon mutagenesis have helped to identify the existence of two T-DNA loci involved in auxin and cytokinin metabolism, tms and tmr. Inactivation of the tms locus produces auxin-dependent tissues which produce shoots (Binns et al 1982), apparently due to alterations in endogenous auxin levels in the tissue (Akiyoshi et al 1983). This locus encodes two enzymes involved in auxin biosynthesis. Tryptophan-2-monooxygenase converts tryptophan to indole-3 acetamide and indole-3-acetamide hydrolase converts this intermediate to indole-3-acetic acid (Thomashow et al 1986, Van Onckelen et al 1986 and Schröder et al 1984).

The cytokinin-requiring cultures produced on mutation of the tmr locus are rooty in morphology (Joos et al 1983) and contain dramatically reduced levels of endogenous cytokinin (Akiyoshi et al 1983). The tmr region codes for a isopentenyl-transferase which synthesises the zeatin precursor, isopentenyl adenine 5-monophosphate (Akiyoshi et al 1984).

Where the integration of the Ti plasmid into the plant cell genome is responsible for the auxin and cytokinin-autonomy expressed in crown gall cultures, the factors underlying the initiation of habituation remain unknown.

1.2 The genetic or epigenetic basis of habituation

A genetic basis for habituation

There is some evidence arising from work on cytokinin-habituated tissues of Nicotiana and Phaseolus that the cytokinin-requirement of plant cells may be under genetic regulation. In crosses between cytokinin-habituated and non-habituated genotypes of both Nicotiana (Meins et al 1983) and Phaseolus (Mok et al 1980), the frequency of cytokinin-autonomy expressed in the F2 populations was found to be 1/4, and in backcross populations, 1/2. These results suggest that cytokinin-requirement in these tissues was controlled by a single pair of alleles. The gene proposed to control cytokinin-requirement in tobacco cultures has been termed habituated leaf (Hl) and has been shown to compensate for the tmr locus in tmr- crown gall mutants, providing further evidence of its possible role in the expression of cytokinin-autonomy in cultured plant tissues (Hansen and Meins 1986, Meins 1987). No sequence homology to the tmr region has, however, been found in non-transformed tobacco cells analysed by Chilton et al (1982) using hybridisation techniques.

Chromosomal variation and habituation

The *in vitro* environment appears to encourage the expression of latent genetic instability in cultured plant cells, with certain genotypes apparently more susceptible than others to the physical and chemical stresses present in the tissue culture environment (Bayliss 1980, Browers and Orton 1982). The culture of dedifferentiated tissues in callus or cell suspensions is a widely acknowledged source of chromosomal instability in tissue culture (Torrey 1967, D'Amato 1977 and Skirvin 1978) and the presence of growth regulators in the culture medium may have an appreciable effect on both the genetic (Bayliss 1973, Orton 1980) and epigenetic stability (Varga et al 1988) of tissue cultures. Chromosomal instability in an *Arabidopsis thaliana* callus culture initiated by Ondréj et al (1984) on a medium containing growth regulators was found to be greater than that found in a spontaneously arising culture which had never been exposed to growth regulators in the culture medium.

Attempts to establish a possible correlation between habituation and alterations in chromosome number have not proved conclusive. Fox (1963) reported markedly higher chromosome numbers in a habituated tobacco strain than in a comparative non-habituated one. The majority of work in this area does not, however, suggest that a relationship exists between habituation and alterations in chromosome number.

Habituated *Helianthus annuus* tissues analysed by

Butcher et al (1975) demonstrated no higher incidence of polyploidy than non-habituated tissues and Binns and Meins (1980) found no significant correlation between chromosome number and cytokinin-habituation in tobacco. The cytokinin-autonomous nodules which arose from tobacco pith tissues analysed by Binns and Meins (1980) were predominantly diploid on removal from cultures grown on an induction medium. Sheridan (1974) reported that an auxin-habituated Lilium longiflorum culture was both diploid and chromosomally stable and Melchers (1971) concluded from his work with tobacco cultures that the chromosome alterations observed in auxin-autonomous tissues were the result, and not the cause of "tumourous" type growth. This suggestion was supported by the observation that plants regenerated from these "tumourous" tissues retained aberrant chromosome numbers.

Somatic mutation

The possibility exists that habituation arises as a result of a somatic mutation in the systems which regulate the metabolism of growth regulators in plant cells. This proposal has been supported by White (1951), De Ropp (1951) and Kandler (1952). Meins and Lutz (1980) originally rejected this hypothesis on the basis of statistical evidence of the incidence of cytokinin-habituation in tobacco, where the average rate of cells transformed per cell generation (4×10^{-5}) was found to be 10^2 to 10^3 times faster than would be expected of a rare, random genetic

mutation.

In contrast to these observations, Meins et al (1983) have now suggested that cytokinin-requirement in tobacco may be a genetic trait controlled by two genetic loci, one of which (Hl-1) may be activated by a mutation to have an oncogenic function sinilar to the tmr locus of the Ti plasmid (Hansen and Meins 1985, Meins 1987).

Everett et al (1981) holds that habituation may arise by either a genetic or an epigenetic mechanism. This theory would account for the often contradictory evidence which exists concerning the induction of habituated cultures in different laboratories. Everett et al (1981), in contrast to the work of Meins and Lutz (1980), observed that the frequency of auxin-habituation in an Acer pseudoplatanus suspension culture to be comparable to the frequency of occurrence of chemical mutagenesis in this tissue and that in this experimental line, auxin-habituation appeared to have arisen as a result of positive selection following a mutation.

Habituation appears to be a progressive condition. Gautheret (1955) was the first to observe that degrees of habituation existed and attributed the nutritional alterations he observed to a "form of enzymic adaptation" in the tissue rather than to a permanent mutation. Meins and Binns (1977) also reported that cytokinin-habituation in tobacco was both gradual and progressive. The tobacco tissues analysed by Meins and Binns (1977) demonstrated variation in the degree of cytokinin-habituation expressed,

with tissues becoming more cytokinin-habituated with time. The progressive nature of the condition only suggests the selection of particular cell types over time, but Meins and Binns (1977) have also recorded reversible shifts between a range of cytokinin-habituated states in tobacco cultures, which lends support to their original theory that habituation in this line of tobacco results from epigenetic alterations.

Evidence of transient genome alterations in habituated tissues

There has been no clear demonstration, to date, of quantitative alterations in the genetic component of habituated tissues but strong evidence does exist to suggest that they do occur. The plant cell genome is capable of undergoing transient, yet stable, alterations in genetic information in response to its environment, including the amplification of gene sequences, deletion of genetic information and hypomethylation of cytosine residues (Walbot and Cullis 1985). The *in vitro* culture of plant tissues inevitably imposes chemical and physical stresses on the cells, one response to which might involve quantitative alterations in genetic information.

Several authors have compared the genetic component of habituated and non-habituated tissues of tobacco. Durante et al (1986) believes that no complex rearrangement of the genome occurs on the conversion of tissues to the habituated state but has provided strong evidence of the

amplification of existing gene sequences in habituated tobacco tissues. Two additional DNA peaks were found by $Ag^{+} - Cs_2 SO_4$ analytical ultracentrifugation in an auxin-habituated tobacco tissue which were not found in non-habituated cultures. One of these extra peaks corresponded to a peak which has been observed in dedifferentiating explants of N. glauca after only 48 hours in culture (Parenti et al 1973).

There is preliminary evidence to suggest that plant growth regulators influence the preferential replication of certain DNA sequences in some species such as Cymbidium (Nägl and Rucker 1972) and cucumber (Kessler 1973). Grisvard et al (1980) reported that variation of the concentration of NAA applied to tissue cultures of Cucumis melo resulted in the appearance of new satellite DNA showing that growth regulators appear to be able to induce genome modifications which could ultimately cause habituation.

The habituated tissues of tobacco analysed by Durante et al (1986) were also found to have a higher DNA-bound ion content than normal tobacco tissues. Guillé et al (1981) has reported that metal ions bound to the DNA at regulatory sequences could induce conformational variations in the DNA which could influence the binding of effector molecules to the DNA and thereby affect gene expression.

Cecchini et al (1983) in a further study of the genetic material of an auxin-habituated tobacco culture, found an apparent correlation between habituation and

hypomethylation. DNA methylation of cytosine residues in specific gene sequences is frequently associated with the inactive state of a gene (Hepburn et al 1987) and hypomethylation of these residues could result in the overexpression of genes, perhaps those involved in the induction of habituation. The levels of 5-methylcytosine measured in tobacco tissues were found to be lower in a habituated culture than a comparative non-habituated one (Citti et al 1983). Cecchini et al (1983) have proposed that hypomethylation could be the mechanism by which the gene sequences involved in auxin and cytokinin biosynthesis are regulated.

1.3 Factors affecting habituation

Endogenous

It is often assumed that habituation arises as a result of prolonged subculturing on a medium containing growth regulators. However, the readiness of certain genotypes to habituate after only short periods in culture demonstrates that this is not necessarily true. Sheridan (1968) initiated a *Lilium longiflorum* culture with a striking readiness to habituate and Mok et al (1980) found that two *Phaseolus* genotypes differed markedly in their capacity for cytokinin-independent growth in culture. *P. vulgaris* exhibited a far greater tendency to habituate than *P. lunatus*. A similar phenomenon was observed in tobacco seedlings by Buiatti et al (1970) with *N. bigelovii* demonstrating a marked readiness to habituate in contrast

to N. quadrivalvis which did not.

Meins and Lutz (1979) have reported that specificity also exists in the explant tissue of tobacco plants in relation to their capacity to exhibit cytokinin-independent growth in vitro. Pith tissues, although capable of habituating readily were initially cytokinin-requiring. Leaf explants were slightly habituated but were still responsive to cytokinin and stem cortex tissues were always cytokinin-habituated. While the phenotypes of leaf and stem remained stable throughout culture, the pith tissues were classed by Meins et al (1979) as a phenotype in which habituation could be induced.

Explant size also appears to influence the capacity of certain tissues to habituate. Habituation in carrot cultures analysed by Duhamet (1955) was found to be very dependent upon the initial size of explant. Meins et al (1980) reported a minimum threshold explant size of 20-30mg below which cytokinin-habituation in tobacco was not expressed. Meins et al (1980) found that the explant size required for the expression of habituation interacted with the nodal position on the plant from which the tissue was isolated. The threshold explant size necessary for habituation to occur was reduced as the nodal position on the plant from which the tissue was isolated moved upwards.

In contrast to these observations, Ondréj et al (1984) reported that large pieces (50mg) of A. thaliana callus expressed less auxin-autonomy than calli propagated in smaller pieces of 10mg.

Effect of exogenous growth regulators

The role of growth regulators in the induction of habituation is an obvious, yet complex, consideration. Auxins are almost routinely required for the induction of callus from most cultured plant tissues (Yeoman and Forche 1980) with few noteable exeptions such as citrus fruit sacs (Kordan 1959). The role of auxin in the general homeostasis of the cell can be easily confused with any apparent role in the establishment of habituation.

Many authors have observed the induction of habituation in tissue cultures after only short-term auxin pretreatments (Ondréj et al 1984, Syõno and Furuya 1972). Buiatti et al (1970) reported that the concentration of exogenous auxin and the length of auxin pretreatment had a considerable effect on the percentage of tobacco tissues which bacame auxin-autonomous. The use of a 20 day auxin (2,4-D) pretreatment in place of a 7 day one was sufficient to habituate all of the N. bigelovii cultures analysed by these authors. Gautheret (1957) demonstrated that an optimum concentration of auxin (3×10^{8}) existed for auxin-habituation in Parthenocissus tricuspidata tissues. This concentration was found to habituate a maximum of 85% of these cultures over a two year period. These observations suggest that the presence and concentration of auxin in the growth medium of cultured tissues does appear to have some influence on the occurrence of habituation.

Meins and Lutz (1980) found that the concentration of kinetin required for cytokinin-habituation in tobacco was

also dependent on the duration of the cytokinin exposure. Meins and and colleagues have proposed that a positive feedback mechanism exists in cytokinin-autotrophic tobacco cultures, where cytokinin regulates its own biosynthesis (Meins and Binns 1978, Meins 1987).

It is also important to consider the interactions which exist between auxins and cytokinins in relation to their influence on the hormone requirements of cultured plant cells. The ability of non-habituated tissues to grow in the absence of auxin when supplemented with high cytokinin concentrations has been reported in tobacco cultures (Syōno and Furuya 1972, Einset 1977). Witham (1968) has reported the opposite occurrence in a soybean culture where high concentrations of 2,4-D allowed the culture to grow in the absence of additional cytokinin. The 2,4-D concentration required for optimum growth in these tissues was found to be lower in the presence of kinetin.

Palni et al (1988) have recently reported that the auxin, NAA, destabilised ^{3}(H) zeatin riboside fed to tobacco pith tissues. Their results suggested that NAA was acting on the cytokinin oxidase system and in this way could affect the cytokinin-requirement of cultured plant tissues.

The in-vitro environment

Many of the physical and chemical conditions in the tissue culture environment could have an influence on the induction of habituation. The *in vitro* environment of

cultured plant cells is standardised as much as is technically feasible, but variation inevitably occurs, accentuated both by time and the closed nature of the system.

Light

Ondréj et al (1984) observed that intense illumination was a requirement for the expresion of auxin-autonomous growth in Arabidopsis thaliana cultures. These cultures required supplements of auxin and cytokinin when grown in the dark. A light-dependent, auxin-habituated Lilium longiflorum culture has also been reported by Sheridan (1974). In contrast to these observations, the isolation of 2,4-D-independent Acer cell lines by Everett (1981) was found to be more frequent from dark-grown cultures. This feature was, however, thought to be a result of a better plating efficiency of cultures in the dark and not related to habituation.

Temperature

Temperature may also have a profound effect on the expression of the habituated phenotype. Binns and Meins (1979) have reported the induction of cytokinin-habituated nodules in tobacco cultures on increasing the culture temperature by 10°C, from 25 to 35°C. These habituated cultures also expressed cold-sensitivity in terms of their cytokinin-requirement by their inability to grow at 16°C in the absence of exogenously applied cytokinin. This cold-

sensitivity was attributed to a decrease in the production of cytokinin in the tissues affecting the putative positive feedback mechanism thought by Meins (1982) to control the maintenance of cytokinin-habituation in tobacco. A similar observation was made by Syōno and Furuya (1971), where the capacity for cytokinin-habituation in tobacco cultures was lost at low temperatures. These cultures grew rapidly in the absence of kinetin at 26°C but growth was inhibited at 16°C, unless the growth medium was supplemented with a cytokinin.

The gaseous environment

There is little information concerning the influence of the gaseous environment on the incidence of habituation. Kövesand Szabo (1987) did not find any correlation between ethylene evolution and habituation. The evolution of ethylene was only found to be enhanced by the addition of auxin to the culture medium of non-habituated tissues.

Inorganic nutrition

Observations by Wood and Braun (1961) that the fortification of Whites medium with the four inorganic salts KCl, $NaNO_3$, $NaH_2PO_4.2H_2O$ and $(NH_4)_2SO_4$ allowed non-habituated Vinca rosea cultures to grow on a medium lacking auxin indicated a major role for inorganic nutrition in the regulation of the metabolism of growth regulators in cultured plant cells. Braun (1969) has proposed that the transformation of a normal to a tumour cell involves an

increase in permeability of the plasma membrane, allowing the increased uptake of ions involved in the biosynthesis of growth factors. The Vinca rosea cultures used by Wood and Braun (1961) may, however, have been partially habituated as the authors results were not repeatable using freshly isolated V. rosea tissues (Wood and Braun 1965). Sogeke and Butcher (1976) were also unable to influence the requirements for growth regulators of V. rosea, H. annuus, N. rustica or D. carota cultures using the inorganic ion supplements of Wood and Braun (1961). Certain habituated tissues were, however, able to grow in the absence of auxin on a high salt medium (Sogeke and Butcher 1976).

Some authors hold that it is not through the activation of biosynthetic systems that inorganic ions act, rather that in affecting membrane permeability the rate of release of ions involved in growth factor metabolism are affected (Stonier and Yang 1971). The uptake of $K^+$and HPO^1 ions was similar in normal and habituated sunflower cultures but the subsequent leakage of these ions from the cells was greater in the habituated tissues. Yoshido and Kohno (1978) reported that auxin-habituated N. glutinosa tissues contained higher amounts of Mg^+, K^+ and P ions than normal tissues and nutrient contents were generally higher in habituated cultures over a 30 day culture period. There was also a marked difference in the organic salt depletion from the growth media of the two tissue types. The mineral composition of the medium supporting normal cells retained 95% of the initial concentrations after 25 days, whilst the

medium of the habituated cultures showed a marked depletion of Ca, HPO ,K, Mg and N to 71, 21, 25, 72 and 39 percent respectively of the original medium concentrations.

1.4 Characteristics of habituated tissues

The biochemistry of habituated tissues

Many physiological and biochemical alterations appear to accompany the conversion of a plant cell to the habituated state. Czosnowski (1952) observed lower levels of protein N, osmotic value, O_2 uptake and protease activity in auxin-habituated Vitis vinifera cultures than in normal cultures. The content of soluble sugars and the activities of amylase, catalase, phosphatase and peroxidase were all higher in the habituated tissue.

Crevecoeur et al (1987) measured a lower level of lignin and cellulose and also an increased water content in auxin-habituated sugarbeet tissues than in a corresponding non-habituated tissue. These authors were trying to establish whether any link existed between the phenomenon of vitrification and habituation. Vitrification is demonstrated by a watery, abnormal appearance to in vitro grown plant tissues (Gaspar 1989) and vitrified tissues contain lower levels of lignin, cellulose and a high water content than normal tissues and therefore some link may exist between the two phenomenon.

Some authors have observed habituated tissues to be of a translucent appearance (eg. Gautheret 1955). Crevecoeur et al (1987) measured chlorophyll levels to be three times

lower in habituated sugarbeet than in normal tissues and three cytokinin-habituated tobacco lines examined by Kaminek and Lustinec (1974) also contained less chlorophyll than a cytokinin-dependent one. Vyskot and Novak (1977), also working with tobacco, found lower levels of chlorophyll in cytokinin-habituated cultures and suggested that a possible link may exist between chlorophyll deficiency and the capacity to grow on a cytokinin-free medium. Such a relationship, if it exists, must be a complex one as auxin-habituated N. glutinosa tissues analysed by Kohno and Yoshida (1977) were found to contain more chlorophyll than non-habituated cultures.

Crevecoeur et al (1987) noted an interesting relationship in the levels of chlorophyll a:b in auxin-habituated sugarbeet calli, where the level of chlorophyll a was found to be higher than that of b. This relationship in chlorophyll a:b was not observed in normal sugarbeet calli and is unusual in higher plant tissues. Again, this feature may not be causally related to habituation as the ratio of chlorophyll a:b in auxin-habituated N. glutinosa cells was not significantly different to that measured in non-habituated cells (Kohno and Yoshida 1977).

Capacity for differentiation and regeneration in habituated tissues

Habituated tissues often exhibit a weak capacity for differentiation (Morel 1948, Henderson 1954). Cytological observations of habituated sugarbeet callus by Crevecoeur et al (1987) demonstrated the total absence of differentiated tracheary elements which were present in the non-habituated tissues. Jones and Scott (1981) made extensive attempts to induce differentiation in a habituated N. tabaccum culture and concluded that this tissue had a lower capacity for regeneration than a comparative non-habituated culture.

In contrast to these observations, occasional shoot production has been observed in certain habituated tissue cultures grown on basal medium (Saunders and Daub 1984). Regeneration of this type is an indication of the inevitable existence of a mixed population of genotypes within these callus tissues. Continuous shoot production by an auxin-habituated culture on basal medium has been reported by De Greef and Jacobs (1979). This shoot production might suggest that the tissue which was differentiated was maintaining the surrounding callus tissues in the production of essential growth regulators. The callus tissue did, however, on removal from the shoot material, retain the capacity to proliferate on basal medium and produce further shoots.

Reversibility of habituation

The potential reversibility of habituation suggests that the condition arises as a result of epigenetic changes in the genetic material. Habituated tobacco tissue cultures have been regenerated into complete plants from both uncloned lines (Sacristan and Melchers 1969) and those of single cell origin (Lutz 1966, Sacristan and Melchers 1977). The habituated cell line (Ta), used by these authors may, however, be of crown gall origin as it appears to contain octopine (see Butcher 1977).

Binns and Meins (1973) regenerated plants from cloned lines of habituated tobacco cultures. Eighty-five percent of these clones were habituated. Sixty-two complete plants were regenerated from 19 different clones, 45 of these flowered and fertile seed was obtained from 10 different clones. Pith tissues of the regenerated plants, on return to culture, were found to be cytokinin-requiring.

There has also been a report of the reversal of auxin-autonomy in tobacco cultures which did not involve regeneration into complete plants (Syõno and Furuya 1974). Auxin-requirement was restored to these habituated tissues by a single 6 week passage on an auxin-containing medium. This form of reversal may, however, merely be a demonstration of the heterogenous nature of the culture.

1.5 Possible mechanisms for the initiation and maintenance of growth regulator autonomy in cultured plant tissues

Metabolic alterations

BUdR tolerance

The observation of Meins (1976) that cytokinin-independent N. tabaccum tissues, sensitive to the thymidine analogue, 5-bromodeoxyuridine (BUdR) were not inhibited by low concentrations of this inhibitor when grown in the presence of kinetin led (Meins 1976) to believe that BUdR blocked the expression of cytokinin-habituation in these cells. In addition to this, the cytokinin-habituated cultures were able to tolerate higher levels of BUdR than non-habituated cultures in the presence of kinetin. Vyskot et al (1977) reported a similar phenomenon, also in N. tabaccum. The tolerance of cytokinin-habituated tissues to BUdR was found to be dependent on the degree of autonomy in the tissue, with the presence of cytokinin significantly increasing tolerance to BUdR.

Kandra and Maliga (1977) did not, however, find any causal relationship between cytokinin-habituation and and BUdR resistence as their results showed that each condition had arisen independently. Everett et al (1981) was also unable to demonstrate any link between BUdR resistence and auxin-habituation in Acer cell lines which were thought to have arisen by a mutation followed by cell selection. A cytokinin-independent soybean line studied by Wang (1979) also demonstrated no more tolerance to BUdR than a

comparative cytokinin-dependent one. The inconsistencies of these results from different laboratories, with repect to BUdR tolerance, do appear to suggest differences in the mechanisms by which auxin and/or cytokinin-habituation arise in cultured plant tissues and dispute any direct link with habituation. The BUdR resistance of one of the tobacco lines studied by Kandra and Maliga (1977) has been shown to be controlled by nuclear gene(s) (Marton and Maliga 1975).

The role of endogenous levels of plant growth regulators in the maintenance of the habituated state

The role of endogenous levels of growth regulators in the initiation and maintenance of autonomous growth in cultured plant tissues has been widely studied. The idea that elevated levels of endogenous auxin and cytokinins are responsible for the growth regulator-autonomy exhibited by both tumourous and habituated tissues has been considered by a number of authors. This theory is supported by evidence that the T-DNA of Agrobacterium tumefaciens integrated into the host cell genome encodes enzymes involved in the biosynthesis of both IAA and zeatin (Morris et al 1982, Thomashow et al 1986) and this has led to the assumption that the levels of endogenous growth regulators would be elevated in these tissues.

Elevated levels of IAA have been measured in certain crown gall (Tandon and Arya 1980, Mousedale 1982) and tumour-prone hybrids of tobacco (Bayer 1965, 1967) in comparison to comparative auxin-requiring tissues.

Increased levels of IAA are not always, however, observed in auxin-autonomous tissues. Weiler and Spanier (1981) found that the auxin content of sterile crown galls was no higher than the tissues from which the cultures were derived and Pengelly et al (1986) measured no increase in the endogenous IAA content of tobacco cells on conversion to the auxin-independent state.

Comparative measurements of endogenous IAA content in auxin-habituated and non-habituated tissues have not been shown to be significantly different in cultures of sugarbeet (Coumans-Gilles et al (1982) and Dioscorea deltoidea (Kutacek et al 1981). In auxin-habituated and non-habituated tissues of tobacco, Koves and Szabo (1987) found that IAA levels were dependent upon the age of the cultures, but the highest levels overall were measured in the non-habituated tissue.

In certain habituated tissues the levels of endogenous growth regulators are extremely low. Nakajima et al (1979) was unable to detect either auxin or cytokinin in habituated tobacco suspension cultures and Hansen et al (1985), using a highly sensitive immunoassay for detection, did not find cytokinin in cytokinin-habituated tissues of tobacco. These results would appear to suggest that a relationship between the levels of endogenous, extractable auxin and cytokinin and growth regulator autonomy has not been conclusively demonstrated. The quantitative analysis of plant growth regulators from plant tissues is a difficult problem due to the extremely low levels of these

substances present, and the problems of analysis are accentuated in the case of auxin by the extremely labile nature of IAA.

The extraction and purification of IAA

A wide variety of analytical methods have been employed for the quantitative analysis of plant growth regulators, including IAA (Horgan 1987). A number of common procedures are followed in any analysis of growth regulators in plant tissues. An initial extract is first obtained from the tissue and this extract is then subjected to a series of purification steps prior to quantification. The initial extraction phase involves maceration of the plant tissue in an excess of solvent of which the most frequently used are acetone and the alcohols, ethanol and methanol (Yokota et al 1980, Sandberg et al 1987). For IAA analysis, anti-oxidant substances such as butylated hydroxylated toluene (Iino et al 1980) and sodium diethyldithiocarbamic acid are frequently introduced to the extraction solvent and to solvents used throughout the purification procedure because of their ability to minimise indole conversions in the extract (Ernsten et al 1986). This is extremely important in the analysis of a compound as labile as IAA and further precautions are often taken during auxin analysis including the avoidance of excess light, O_2, high temperatures and extremes of pH (Martin et al 1986, Sandberg et al 1987 and Yokota et al 1980) in order to minimise chemical alterations to the in situ

levels of IAA.

The isolation of IAA from the crude plant extract often involves a number of different purification techniques. The current trend is moving away from extensive purification and the extensive use of solvents (Chen et al 1988). Various forms of chromatography are used in the purification of IAA from crude plant extracts (Sandberg et al 1987) including PVP (Martin et al 1986), Sephadex LH20 (Wyndaele et al 1985, 1988), Sephadex G25 (Sweetzer and Swartzfager 1978), DEAE Sephadex A-25 (anion-exchange) (Pengelly et al 1981, 1986) and TLC (Iino et al 1980, Martin et al 1986). A combination of these chromatography steps are usually employed in order to obtain an extract of sufficient purity for quantification.

Before the advent of advanced physicochemical techniques for the detection and quantification of plant growth regulators including IAA, bioassays were the main method of detection. The first assay for auxin activity, the Avena coleoptile assay, was developed by Went (1928) and several other bioassays have since been devised. These bioassays have been gradually replaced by chemical identification, as new techniques have become available. Physicochemical techniques are more sensitive for the detection of IAA than methods based on biological activity, as several other compounds present in plant tissues exhibit auxin activity, including other indoles.

A more specific assay for the detection of IAA was developed by Stoessl and Venis in 1970. This assay involves

measuring the fluorescence produced by the conversion of IAA to 2-methylindolo-2,3:3,4-pyr-6-one (2-MIP) on reaction with acetic anhydride in the presence of trifluoroacetic acid. This assay is also subject to inaccuracies arising from contamination as some compounds cause fluorescence similar to IAA on this reaction, such as 4-Cl-IAA and 5-OH-IAA. Problems of contamination can, however, be minimised by separation steps prior or subsequent to derivatisation (Iino et al 1980, Blakesly et al 1984).

HPLC is now widely employed for the quantification of plant derived IAA. HPLC offers good resolution, rapid analysis and a large sample capacity. Of the detectors available for HPLC, fluorescence detection is perhaps the most specific for IAA (Burnett and Audus 1964). Crozier et al (1980) has reported the detection of as little as 1pg of IAA using a modified fluorescence detector. HPLC-F is superior to U.V. detection for IAA (Sweetzer and Swartzfager 1978, Akiyama et al 1983). An alternative detector for HPLC is the electrochemical detector which offers a sensitivity intermediate between U.V. and fluorescence (Law and Hamilton 1982, Sweetzer and Swartzfager 1978).

Gas chromatography has also been used for the detection of IAA in plant tissues (eg.Moloney et al 1983). For GC analysis samples must be converted to a volatile derivative, mainly to the methyl ester or heptabutyryl methyl ester (Hofinger 1980). The flame ionisation derector (FID) is the most common detector used for GC analysis but

lacks specificity for IAA. More sensitive detectors available for GC are electrochemical detectors (ECD) and nitrogen phosphorous detectors (NPD) (Hunter 1986) which offer equivalent sensitivity to HPLC-F in the detection of IAA (Hunter 1986).

The introduction of immunoassays for the detection of plant growth regulators was a major advance in terms of specificity but this technique is not without problems of cross contamination. The small size of IAA requires the compound to first be covalently bonded to a protein in order to induce an antigenic response in a host animal. Pengelly and Meins (1977) were the first to report a radioimmunoassay (RIA) for IAA where the auxin was covalently bonded to bovine serum albumin via the indole nitrogen. Pengelly et al (1981) reported problems caused by the presence of pigments and other interfering compounds in their immunoassay.

Physicochemical techniques for the detection of IAA require validation to demonstrate that the substance isolated and quantified is the desired compound. A GC coupled to a mass spectrometer is the ultimate tool for the identification and quantification of IAA and is being increasingly used (Chen et al 1988) for this purpose. GC-MS is not, however, a readily available technique and most experimenters have to rely upon more than one mode of detection as a means of validation.

Metabolism of endogenous IAA

The mechanism by which steady state auxin levels are maintained in cultured plant cells has attracted much attention in relation to auxin-autonomous growth. The overall levels of free IAA available in plant cells is the result of a balance between the mechanisms which produce IAA and those which remove it from the IAA pool. IAA may be produced from either de novo synthesis from the major precursor tryptophan (Schneider and Wightman 1974, Black and Hamilton 1976) or released from the hydrolysis of IAA conjugates (Cohen and Bandurski 1984).

Biosynthesis of IAA

Tryptophan is widely acknowledged as the major precursor of IAA in plant tissues (Schneider and Wightman 1974). The majority of plant tissues studied appear to follow a pathway of IAA biosynthesis from tryptophan via indolepyruvic acid (Cohen and Bialek 1984, Atsumi 1980, Gibson et al 1972 (a) and (b)). This compound is an extremely unstable intermediate produced by the activity of tryptophan aminotransferase, an enzyme which is broadly specific for the transamination of aromatic amino acids and L-aspartic and L-glutamic acids (Schneider and Wightman 1974). This enzyme is widely distributed in plants (Truelsen 1973, Wightman 1973, Lui et al 1978).

Indoleacetaldehyde, a major intermediate in IAA biosynthesis is formed from indolepyruvic acid by the enzyme indolepyruvate decarboxylase. This enzyme has been

found in tobacco shoot extracts (Gibson et al 1972 (b)) and mung bean seedlings (Wightman and Cohen 1968). The conversion of indoleacetaldehyde to IAA is thought to be controlled by either an NAD dependent indoleacetaldehyde dehydrogenase which has been found in mung bean seedlings (Wightman and Cohen 1968) or an oxygen requiring indoleacetaldehyde oxidase which has been identified in oat coleoptiles (Rajagopal 1971) and tobacco callus (Liu et al 1978).

An alternative or additional pathway for IAA biosynthesis from tryptophan via tryptamine has been reported in certain plant species. Tryptamine has been formed as a metabolite of ^{14}C tryptophan in feeding studies in tobacco terminal bud tissue (Phelps and Sequiera 1968). Tryptamine is produced from tryptophan by the enzyme L-tryptophan decarboxylase found in tomato and barley shoots (Gibson 1972 (a) and (b)). This enzyme is more specific for tryptophan than the transaminase but is not as widely distributed in the plant kingdom. Oxidative deamination converts tryptamine to indoleacetaldehyde.

Some authors have shown that indoleacetaldoxime is a potential intermediate of IAA biosynthesis in certain plant tissues in radiolabelled tryptophan feeding studies (Helmlinger et al 1985, Rausch et al 1985). The proposed pathway from tryptophan via indoleacetaldoxime is thought to involve indoleacetonitrile as a second intermediate which is hydrolysed to IAA by the enzyme nitrilase (Sembdner et al 1980). This enzyme is of very limited

distribution in plants and has been observed mainly in Cruciferae (Sembdner et al 1980).

The pathway of IAA biosynthesis in plant tissues infected by the bacteria Agrobacterium tumefaciens and Pseudomonas savastonii involves the formation of indoleacetamide as an intermediate (Van Onckelen et al 1985, Thomashow et al 1986). This T-DNA induced pathway appears to be present in addition to the natural pathways followed in Agrobacterium transformed plant tissue via indolepyruvic acid (Atsumi 1980), tryptamine (Rausch et al 1985) or indoleacetaldoxime (Rausch et al 1985). Figure 1.1 summarises the proposed pathways of IAA biosynthesis in plant tissues.

5-MT resistence

The resistence of various various habituated and crown gall cultures to the tryptophan analogue, 5-methyl tryptophan (5-MT), has been suggested as a mechanism by which these tissues exhibit auxin-independent growth. The altered sensitivity of anthranilate synthetase, a key enzyme in the biosynthesis of tryptophan, to feedback inhibition causes the accumulation of tryptophan in cells which are 5-MT resistant (Widholm 1972).

Sung (1979) reported that tryptophan accumulation resulted in elevated levels of endogenous IAA in 5-MT resistant carrot cultures and that these tissues were also auxin-habituated. The observation by Widholm (1977) that although 5-MT resistant cells of tobacco and carrot

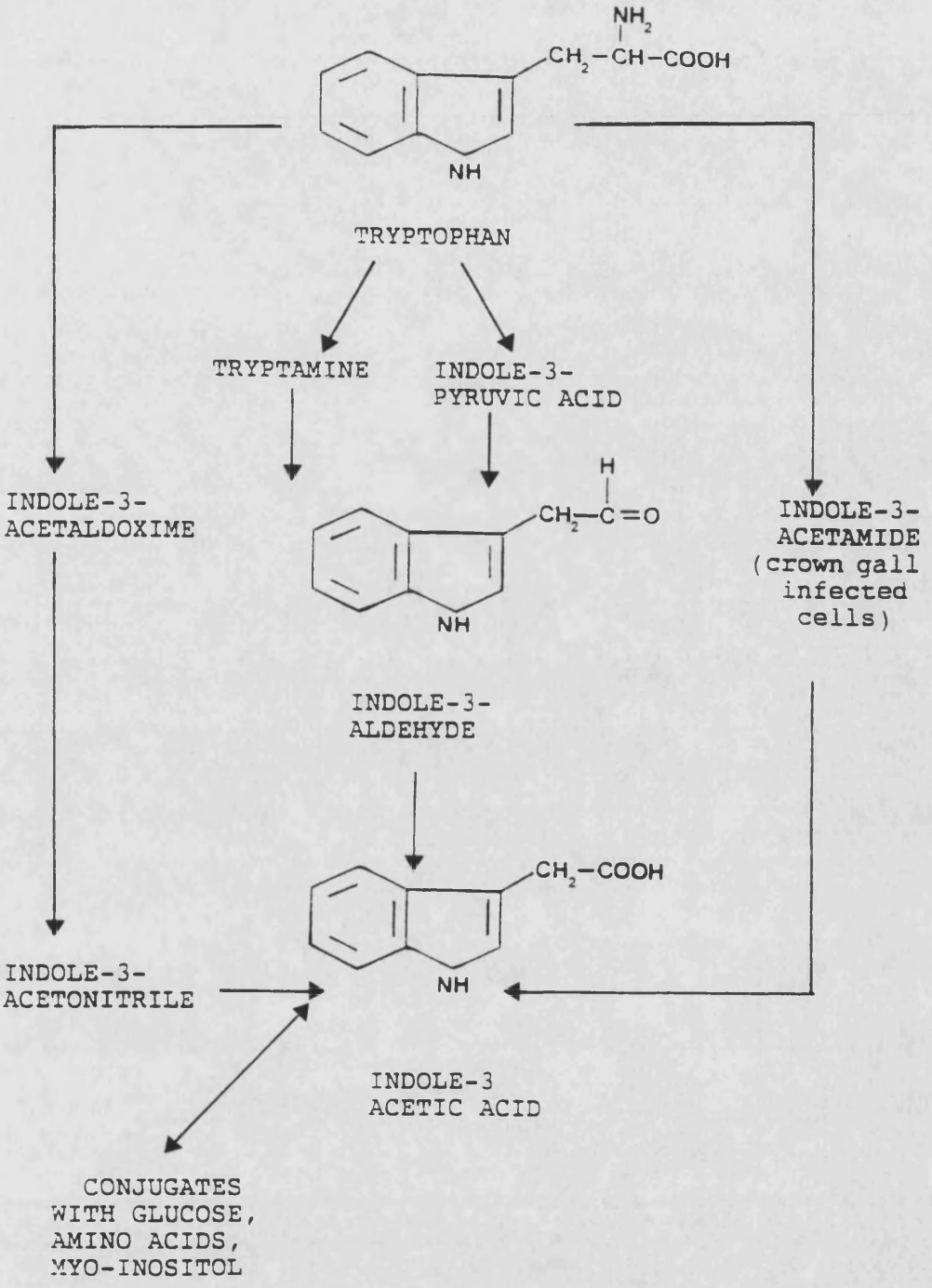


Figure 1 : Pathways of IAA biosynthesis in plants (Roberts and Hooley 1988).

accumulated more tryptophan than non-resistant cells, only a proportion of these cultures were auxin-habituated demonstrated that a causal relationship between 5-MT resistance and auxin-habituation did not appear to exist. This observation may be related to the inability, to date, to demonstrate a correlation between the elevation of levels of endogenous IAA and auxin-autonomy.

Conjugation of IAA

Plant tissues appear to contain much of their IAA in the form of bound conjugates (Bandurski and Schulze 1977). These ester and amide conjugates are formed by the covalent linkage of IAA to molecules such as glucose, myoinositol, aspartic acid and other amino acids (Cohen and Bandurski 1982). Most of the knowledge concerning IAA conjugates stems from the work of Bandurski and colleagues on Zea mays seeds and seedlings, with a lack of information of different developmental growth stages and other plant species.

Several possible functions have beeen reported for these IAA conjugates. Cohen and Bandurski (1982) have suggested that IAA conjugates represent a storage form of IAA in Zea mays seeds and seedlings, as the rate of de novo synthesis measured from tryptophan was very low in these tissues (Hall and Bandurski 1978, Epstein et al 1980). Conjugates have also been shown to protect IAA from peroxidase attack (Cohen and Bandurski 1978), a function which would be useful in a possible role as a transport

form of the auxin. The binding of IAA to larger molecules as conjugates may also be a mechanism of withstanding toxic levels of auxins applied to plant tissues. Nowacki and Bandurski (1980) have reported that the application of exogenous IAA led to the formation of auxin conjugates in Zea mays seeds.

One of the most important roles of IAA conjugates, with relevance to this present discussion, could be in the regulation of the levels of free IAA in plant tissues. The apparent ability of conjugates to withstand peroxidase attack is an important consideration here. The synthesis and hydrolysis of IAA conjugates may be a mechanism by which IAA levels are regulated in plant tissues rather than the de novo synthesis of the auxin. Alterations in the ratio of free:bound IAA measured in plant tissues in response to environmental stimuli demonstrates the involvement of these conjugates in the control of IAA levels. A 30% reduction in growth rate was measured in Zea mays seedlings in response to a growth-inhibitory light flash and this was accompanied by a 40% decrease in the concentration of free IAA and an increase in esterified forms of the auxin (Bandurski et al 1977). It would seem that IAA conjugates play a major role in the maintenance of intracellular IAA concentration in seeds and seedling tissues and their possible role in the control of intracellular IAA levels in other plant tissues including dedifferentiated tissue cultures cannot be disregarded.

The catabolism of IAA

There are two known types of enzymic oxidative processes involved in the catabolism of IAA (Reinecke and Bandurski 1987). The first involves a decarboxylating enzyme with apparent peroxidase activity (Galston et al 1953, Kenten 1955). This pathway of IAA catabolism has been widely studied *in vitro* using purified peroxidases (Hinman and Lang 1965). The rate of enzymic decarboxylation may be increased by Mn and monophenols (Schneider and Wightman 1978). Peroxidases in tomato plants have been shown to be H_2O_2 requiring (Kokkinakis and Brooks 1979) and H_2O_2 can also reduce the lag period for oxidation in other tissues (Schneider and Wightman 1978). The decarboxylation of the side chain from IAA and the resulting oxidation converts IAA to several metabolites of which 3-methyleneoxindole, 3 hydroxy-methyloxindole, indole-3-aldehyde and indole-3-methanol are the major products (Hinman and Lang 1965).

The second form of enzymic degradation of IAA does not involve decarboxylation (Reinecke and Bandurski 1987). This pathway has been demonstrated in *Zea mays* (Bandurski 1984), *Pisum sativum* (Davies 1973) and *Vicia fabia* (Tsurumi and Wada 1985). The proposed products of this pathway are oxindole-3-acetic acid and dioxindole-3-acetic acid (Reinecke and Bandurski 1987) and a direct relationship between IAA and these compounds has been demonstrated using radiolabelled feeding studies in both *Zea mays* (Reinecke and Bandurski 1987) and *Vicia fabia* (Tsurumi and Wada 1980). Oxidation irreversibly removes IAA from plant

tissues in the formation of various metabolites and derivatives. The exact mechanisms of enzymic oxidation and the location of this activity are unknown and further information is required before the importance of this form of metabolism to the overall regulation of endogenous IAA levels and auxin-independent growth can be determined.

Both Coumans-Gilles et al (1982) and Crevecouer et al (1987) have reported lower levels of peroxidases in auxin-habituated sugarbeet tissues in comparison to non-habituated tissues. Kevers et al (1982) observed that peroxidases were released from sugarbeet cultures. The levels of peroxidases released were found to be in proportion to the endogenous levels in the tissue and the release of the enzymes was found to be promoted by calcium ions, moreso in auxin-habituated cultures. These results suggest that the regulation of endogenous IAA levels may occur via alterations in the rate of IAA catabolism.

Auxin protectors

Auxin protector substances are thought to be involved both in the control of endogenous auxin levels (Atsumi and Hayaisha 1978) and in auxin-autonomy (Stonier 1971). These substances have not been fully characterised but have been shown to induce a lag in the destruction of IAA and not an overall reduction in rate and are therefore not enzyme inhibitors. High protector levels in sunflower tissues have been associated with juvenile tissue (Stonier and Yang 1967) and wounded internodes infected by a virulent strain

of Agrobacterium tumefaciens (Stonier 1969). An increase in auxin protectors was observed to accompany the accumulation of endogenous auxin during the transitional stage in sunflower crown gall tissues (Atsumi and Hayaisha 1978). The authors also reported highest auxin destruction activity at maximum levels of endogenous auxin in the tissue and proposed that auxin itself regulated its own endogenous IAA concentration by a form of negative feedback mechanism.

Coumans-Gilles et al (1982) measured higher levels of both peroxidases and protectors in a non-habituated sugarbeet calli than in an auxin-habituated one and these factors were thought by the authors to be the cause of equivalent IAA levels measured in the two tissues. Weis (1967) reported greater protection of IAA from destruction in non-habituated tobacco tissues than in comparable habituated cultures. Syõno (1979), however, measured an increase in the protection of IAA from destruction activity during the induction of auxin-autonomy in tobacco tissues and an alteration in the rate of IAA destruction in plant tissues could be important to the induction and maintenance of auxin-habituation.

Auxin receptors

It may not be the levels of endogenous growth regulators which are important in the expression of autonomous growth, but rather the number of receptor sites available for these growth regulators in plant tissues (Trewavas 1982). Auxin-binding sites have been reported in a number of plant species (Libbenga et al 1986) both monocotyledonous, such as the well characterised membrane auxin binding protein (ABP) of maize (Dohrmann et al 1978, Venis 1987, Löbler and Klämbt 1987, Inohara et al 1989) and dicotyledonous including tobacco (Maan et al 1983, Starling et al 1986, Barbier-Brygoo et al 1989), Pisum (Jacobsen 1982) and soybean (Williamson et al 1977).

There is evidence that auxin-binding sites are influenced by the surrounding growth conditions of the tissue. Vreugdenhill et al (1981) reported that the number of auxin-binding sites in batch cultures of tobacco varied with the culture cycle. Starling et al (1986) found that, although the levels of salts and sucrose in the medium affected the growth rate of tobacco callus cultures, they had little effect on the on the number of specific binding sites for auxin in the tissue. These results suggest that the observations of Vreugdenhill et al (1981) could perhaps be related to variations in growth factors other than organic nutrients, possibily endogenous growth regulators.

Growth regulators also appear to have an influence on the appearance of auxin-binding sites. Trewavas (1980) observed the stimulation of auxin-binding sites in

Jerusalem artichoke tuber tissues on incubation on a 2,4-D containing medium. A further auxin-binding site, apparently influenced by 2,4-D, has been described by Van der Linde et al (1985). This site appeared in tobacco pith explants after only one day in culture on a medium containing 2,4-D and was thought by the authors to be involved in auxin-regulated cell division. This was due to its ability, in combination with auxin to elevate RNA levels in isolated nuclei. A second plasmalemma bound auxin binding site was reported in this tissue, present only when the tissue was cultured on a medium containing both NAA and kinetin. This binding site was thought by Van der Linde et al (1985) to be involved in regeneration because of its appearance on regeneration induction medium and its absence on proliferation medium containing 2,4-D as the only additional growth regulator.

There has not been, to date, any unequivocal evidence for the existence of receptors for plant growth regulators. One of the clearest demonstrations yet, of a plasmalemma bound auxin receptor in plant tissues is that reported by Barbier-Brygoo et al (1989) in tobacco mesophyll protoplasts. There is evidence that an ATPase is involved in an auxin-induced variation in the transembrane potential difference (Em) as the binding of NAA to the receptor leads to the activation of a proton pumping ATPase. Inohara et al (1989) have recently purified and cloned an auxin-binding protein found in the endoplasmic reticulum (E.R.) of maize shoots. The primary structure of this protein has been

elucidated and the COOH terminal sequence was found to be common with that proteins located in the lumen of the E.R. of animal cells suggesting that this is also the location of this auxin-binding protein in plant cells.

The mechanism by which the binding of auxin to a receptor could elicit the intracellular chain of reactions leading to and accompanying growth is unclear. Theologis (1986) proposes that the primary action of the auxin-receptor complex is to directly induce mRNA synthesis of a specific set of auxin-regulated genes involved in growth. There is evidence of the induction of specific sets of mRNA in auxin treated soybean (Hagen et al 1984) and pea epicotyl segments (Theologis 1986) accompanying cell elongation in these tissues. Similar auxin-induced mRNA's have been reported by the addition of auxin to auxin starved in vitro cultured cells of soybean (Bevan and Northcote 1981) and tobacco (Van der Zaal et al 1987) suggesting their involvement in auxin-induced cell division in these tissues.

Several auxin-induced mRNA's, which are highly specific to auxin, have now been identified and their corresponding genes characterised (Guilfoyle and Hagen 1988). Most of these mRNA's are detected between 15 to 30 minutes after auxin application. However, the function of these auxin-regulated gene products remains unclear.

It is possible that the auxin-receptor complex may act directly on the DNA in a similar manner to steroid hormones in animal cells in which a hormone-receptor complex

interacts with a specific region of DNA on hormone-induced genes. However, many authors believe that secondary messengers are involved in the transduction of signals from auxin bound to its receptor at the plasmalemma to the site of action.

In animal cells, both cAMP and Ca^{2+} play a major role in cell growth by regulating a number of enzymes involved in critical cell processes (Krebs and Beavo 1979). In plant cells, whilst the phosphorylation of several polypeptides in Zucchini hypocotyl membranes are reported to be regulated by Ca^{2+}, the involvement of cAMP in the activation of protein kinases in plant cells remains elusive (Salimath and Marre 1983). Ca^{2+} has been proposed by a number of authors as the most likely candidate for the transduction of auxin signals in plant cells (Hepler and Wayne 1985, Gilroy et al 1987, Dieter 1984). Cunninghame and Hall (1986) demonstrated the inhibition of auxin-induced growth in Pisum sativum stem segments by the use of Ca^{2+} uptake blockers. A similar inhibition of auxin effect on Ca^{2+} depleted maize roots was relieved on the re-introduction of Ca^{2+} to the tissues (Hasenstein and Evans 1986).

There is also evidence that auxin can stimulate the release of Ca^{2+} ions from the vacuole (Hertel 1983), E.R. (Brummer and Parish 1983) and the microsomes (Buckhout 1984) of plant cells. Plant cells contain efficient sequestration mechanisms enabling the maintenance of low levels of cytosolic Ca^{2+} in comparison to the levels present in the vacuole and cell wall (Hepler and Wayne 1985)

because high levels are cytotoxic (Gilroy et al 1987). Low levels of Ca^{2+} are maintained in the cytoplasm by the removal of Ca^{2+} to by ATPases and Ca^{2+}/H^{+} antiport (Macklon 1984, Bush and Sze 1986). The increase of cytosolic levels of Ca^{2+} caused by auxin is thought to be accompanied to the binding of Ca^{2+} to calmodulin, a Ca^{2+} binding enzyme regulator. Calmodulin, which is activated on the binding of auxin has been widely reported in plant tissues (Dieter 1984, Hepler and Wayne 1985). This activated form of calmodulin may regulate the activity of several enzymes in plants (Dieter and Marme 1984) including an NAD kinase (Anderson and Cormier 1978) and ATPases (Dieter and Marme 1981).

The use of calmodulin-binding antagonists and inhibitors has identified auxin-requiring processes which may also be Ca^{2+}/calmodulin-dependent. These compounds have blocked auxin-dependent growth in wheat coleoptiles (Elliott et al 1983) and elongation in oat coleoptile segments (Ragothama et al 1985). Roberts and Baba (1987) also reported the use of these inhibitors to demonstrate Ca^{2+}/calmodulin involvement in auxin-induced xylogenesis of Lactuca pith explants.

Ca^{2+} may not be the only messenger which responds to auxin in plant tissues. Inositol-1,4,5-trisphosphate (IP3) also induces the release of Ca^{2+} from permeabilised animal cells and microsomal preparations (Berridge 1984). In plant cells auxin induces the turnover of phosphoinositides in membranes (Morre and Ackerman 1984). Drobak and Ferguson

(1986) suggested that auxin causes an increase in cytosolic Ca^{2+} levels by increasing the inositol-3-phosphate released from enhanced phosphoinositide turnover. They demonstrated that inositol-3-phosphate effected the release of Ca^{2+} from the microsomes of Zucchini hypocotyls. Recently, Gilroy et al (1990) reported that the release of inositol-3-phosphate caused an influx of Ca^{2+} into the cytosol of guard cells of Commelina communis which was followed by stomatal closure. This evidence suggests that Ca^{2+} and inositol-3-phosphate act as secondary messengers in plant cells.

1.6 Auxin and the control of plant cell proliferation

Auxin and the cell cycle

The idea that auxin and cytokinin regulate the cell division cycle in plants through control at specific points in the cell cycle is widely accepted. Two main regulatory points are thought to exist, a G1/S transition and a G2/M transition stage, in a set sequence of events in the cycle (Van't Hof and Kovacs 1972, Van't Hof 1985). Evidence for the direct involvement of auxin and cytokinin in the plant cell cycle is, however, lacking mainly due to the difficulties of technically isolating this the cell cycle from the vast network of intracellular reactions in which these growth regulators appear to be involved. Reports do, however, exist of the direct involvement of auxin in DNA synthesis and cytokinin in mitosis in tobacco (Simard 1971) and soybean cultures (Fosket and Short 1973). Bayliss (1985) has highlighted the lack of clear evidence of the

direct involvement of auxin and cytokinin in the regulation of the cell cycle in cultured plant tissues in a review of this subject.

Plant tissue culture has been of great value in the study of the cell cycle in plants (Gould et al 1974). Synchronised cell cultures, obtained both by manipulation of growth medium constituents and the environment of the cultured cells, have been particularly useful in attempting to pinpoint possible regulatory stages in the cell cycle (Yeoman and Forche 1980). Partial synchronisation of cell cultures has been obtained by auxin deprivation (Nishi et al 1977), cytokinin deprivation (Jouanneau 1971) and through more complex pretreatments involving light and irradiation (Nishinari 1976). There is also the natural synchrony of the plant cell cycle which is demonstrated in dormant tuber tissues such as those of Jerusalem artichoke (Yeoman and Mitchell 1970).

Some authors believe that auxin-deprived tissues are halted in the G1 phase of the cell cycle (Nishi et al 1977, Yeoman and Mitchell 1980). The involvement of auxin in the transition from the G1 to the S phase has been demonstrated in Jerusalem artichoke tuber explants, where the application of auxin causes the G1 arrested cells to proceed synchronously through at least one cell cycle (Yeoman and Mitchell 1970). RNA and protein synthesis are repressed and then stimulated prior to DNA synthesis and this increased respiration is generally thought to be under the regulation of the added 2,4-D (Yasuda et al 1974). It

is not clear whether this regulation is direct or indirect.

Auxin involvement in cell cycle regulation may not be solely at the G1/S transition stage. There is also evidence for auxin involvement in mitosis (Das et al 1956, Naylor et al 1954, Gamburg 1982). Recently, Baizi and Jaminez (1989) have demonstrated that the synthetic auxin MCPP stimulated G2 arrested cells of embryonic maize tissue to enter cell division. Gamburg (1982) reported that auxin controlled two separate stages of the cell cycle in a cytokinin-autonomous tobacco cell culture. The majority of cells halted prior to DNA synthesis when deprived of auxin. A short term auxin pretreatment permitted the cells to proceed through DNA synthesis with a further addition of auxin required for mitosis to take place.

Further evidence for the involvement of auxin in the regulation of cell division is provided by the quantitative evidence of Leguay and Guern (1977) who observed that the onset of the stationary phase in cultured Acer cells occurred when endogenous 2,4-D levels fell below a threshold value necessary for cell division to proceed. Robinson (1982) also found that endogenous IAA levels in this tissue correlated with the number of cells in mitosis. These findings strongly suggest that the concentration of endogenous auxin is important to the regulation of cell division in plants.

The role of growth regulators in cell growth and division may not, however, be as direct as the previous discussion implies. Trewavas (1985) has proposed that a

number of nutritional and environmental conditions are capable of influencing cell division in plant tissues and that the role of growth regulators may not be a specific one in governing the cell cycle but may only permit cell division to proceed.

Some authors believe that the cell division cycle in plants is governed indirectly via the rate of cell growth, which is in turn determined by the the availability of growth regulators and nutrients (Mitcheson 1977, Cavalier-Smith 1985). This hypothesis suggests that nutrient uptake by the cells would be the most efficient stage at which regulation could occur.

Rausch et al (1984) has proposed that a primary role of auxin in the auxin-independent growth of tobacco crown gall tissues could be in the regulation of solute uptake by these cells. The uptake of solutes from the growth medium is of critical importance to a rapidly dividing tissue such as a tumour (Braun 1969) and regulation of nutrient uptake at the plasma lemma could ultimately be the limiting factor in cell division. This theory is similar to the ideas of Braun and Wood (1962) in suggesting that an increased uptake of solutes was responsible for the stimulation of the regulatory mechanism of growth control in tumourous tissues.

There are other indirect mechanisms by which plant cell growth could be regulated. The modulation of cell proliferation by auxin may be effected via control of the rates of intracellular precursor modification and transport

of these precursors to the sites of macromolecular assembly prior to DNA synthesis. Alternatively, direct regulation of DNA synthesis may occur, with auxin involved in the regulation of the incorporation of nucleosides into DNA.

There is presently, however, too little evidence to accurately speculate on the exact nature of auxin involvement in plant cell growth, but further evidence concerning auxin receptors (Rudelscheim et al 1987), secondary messengers and the function of auxin-regulated gene products (Guilfoyle and Hagen 1988) could clarify whether this involvement is direct or indirect and provide information regarding the mode of action of auxin at the molecular level.

1.7 Aims of the current project

The aim of this present work was to investigate the biochemical role of endogenous IAA in the proces of auxin-habituation. The significance of endogenous levels of auxin and cytokinin to the expression of growth regulator autonomy in cultured plant tissues is a key question which requires clarification. This present study has therefore concentrated on this issue by comparing both the level and metabolism of endogenous IAA in both auxin-habituated and non-habituated tissues.

In addition to any role endogenous levels of auxin may have in determining the auxin-habituated state, IAA may also have a more direct role in the control of cell division in plants. Auxin-habituated tissues can be

employed to examine the requirement for auxin of primary growth processes such as DNA synthesis, by monitoring DNA synthesis in the presence and absence of auxin antagonists, which appear to be able to modulate endogenous levels of IAA. The effect on DNA synthesis of a reduction in endogenous IAA levels can then be monitored in tissues requiring no additional auxin for growth.

It is hoped that these studies might contribute to a better understanding of the role of endogenous IAA in the regulation of plant growth both *in vitro* and *in vivo*, and in particular in relation to auxin autonomous growth in cultured plant tissues.

Chapter 2

Growth regulator requirements for the initiation of callus from leaf explants of Lactuca and the induction of habituated tissues

2.1 Introduction

The isolation of habituated cultures first requires the initiation of callus from excised plant parts cultured *in vitro*. The dedifferentiation of plant cells to form callus is a natural response to wounding in intact plants, a response which may also be initiated and maintained *in vitro* by the addition of growth inducing substances to the culture medium of tissue explants. Callus formation involves the induction of cell division in quiescent tissues under the influence of growth regulators (Yeoman and Forche 1980).

Growth regulators are almost certainly important in the induction of habituation (Gautheret 1955, Meins and Binns 1978, Bennici 1972) and their involvement at this initial stage of the induction of cell division and callus formation could be critical to the ultimate ability of cultures to habituate. The work in this Chapter concerns the initiation of callus from leaf explants of *Lactuca sativa* (var. L.) and the subsequent isolation of habituated tissues from these calli. The requirement for auxin and cytokinin in callus initiation from this *Lactuca* leaf tissue was studied.

2.2 Materials and methods

Plant material

Seeds of Lactuca sativa (var.L."Lobjoits Green Cos", Suttons Seeds Ltd.) were sown in Levington seed compost and the seedlings transferred into 9cm plastic pots containing Levington potting compost. The plants were grown in the glasshouse for a period of six weeks. Healthy leaves were removed from the plants to provide explant material for callus induction.

Preparation of explants for in vitro culture

Lettuce leaves were surface sterilised for 10 minutes in sodium hypochlorite solution (0.1% available chlorine) agitated by a magnetic stirring bar. The leaves were then removed from the sterilant and rinsed three times in sterile distilled water. Discs of 10mm diameter were cut from the leaves using a sterile cork borer (No. 6, 10mm i.d.) and placed, using sterile forceps, into 100ml sterile plastic petri jars (Serilin Ltd.) containing approximately 25ml of Murashige and Skoog medium (Murashige and Skoog 1962, pH 5.5, 0.5% agar, 2% sucrose) prepared from stock solutions. The culture medium contained both NAA (1mg/l) and kinetin (0.5mg/l). The culture jars were sealed with transparent petri dishes (5 x 3cm, Sterilin Ltd.) and a thin layer of parafilm (American Can Company, Greenwich CTO6830) was used to seal the vessels. The cultures were placed in a Gallencamp incubator at 25°C in continuous light of 685 μ mol m s^{-1}. The procedures described above and

all subsequent manipulation of *in vitro*-grown plant tissues were undertaken in a laminar air flow cabinet.

Callus culture

After three to four weeks in culture, callus tissue formed mainly around the periphery of the explants was excised from the leaf discs using a sterile scalpel and forceps and transferred to fresh medium (Murashige and Skoog, pH 5.5, 0.7% agar, 2% sucrose) containing NAA (1mg/l) and kinetin (0.5mg/l). Callus tissues were cultured in 100ml plastic petri jars sealed with transparent lids and incubated under the standard conditions described above. These tissues were subcultured at three week intervals by transferring 50mg explants of callus to fresh culture medium.

Assessment of callus growth

Growth of callus tissues was assessed by measurement of fresh weight increase. Explants were weighed at the beginning of a culture period and re-weighed after three weeks in culture. The growth of the tissues was expressed in terms of the tissue doubling constant, k.

$$k = \frac{\ln (Wt/Wo)}{t}$$

where:-

k = tissue doubling constant

Wo= initial fresh weight of the tissue

Wt= fresh weight at time, t

t = time in culture

The time taken in days for the tissue to double in fresh weight, td, could then be calculated by the following equation:

$$td = \frac{0.69}{k}$$

Origin of auxin-habituated Lactuca callus

The auxin and cytokinin-habituated Lactuca tissue used throughout this work was isolated by Savigear (1985). This habituated tissue arose on the surface of an organogenic, auxin-requiring callus originated from leaf discs by the method described above. On transfer of this organogenic tissue to a medium without auxin or cytokinin, the culture became overgrown with disorganised tissue. The habituated phenotype of this disorganised tissue was demonstrated by the removal and subsequent proliferation of this tissue on a medium lacking auxin and cytokinin for more than three subculture passages (> 9 weeks). This auxin habituated tissue was maintained on Murashige and Skoog medium (pH 5.5, 0.7% agar, 2% sucrose) without additional growth

regulators for at least 1 year prior to the start of this project. The growth of this tissue over a period of 63 weeks (21 growth passages) is presented in Figure 2.1 and Plate 1 shows the phenotype of this habituated callus after 21 days of culture on basal medium. The doubling time of this habituated culture was calculated as 3.9 days (Appendix A).

Origin of non-habituated Lactuca callus tissue

A non-habituated, auxin-requiring Lactuca callus tissue was isolated in this present project from leaf discs of the same genetic source as the habituated culture (var. L. "Lobjoits Green Cos"). This tissue was isolated by the method of callus induction described above and was used as a comparative experimental tissue to the habituated line. Plate 2 shows this non-habituated tissue after 21 days growth on a maintenance medium containing NAA (1mg/l) and kinetin (0.5mg/l).

The growth of this non-habituated tissue over a period of 63 passages is presented in Figure 2.2. The doubling time of this tissue when grown on a medium containing NAA (1mg/l) and kinetin (0.5mg/l) was 5.1 days (Appendix A). This line of tissue, unlike the habituated one, demonstrated organogenic potential (root production) when cultured on the medium containing NAA and kinetin for longer than three weeks without subculture (Plate 5). Root production was not, however, observed during the three week culture passages.

Assessment of growth regulator dependence/independence

In order to assess the degree of habituation or growth regulator dependence of the habituated and non-habituated callus tissues, 50mg explants of each tissue type were transferred to fresh medium with or without growth regulators as appropriate, and the subsequent growth of the tissue analysed by fresh weight increase, as described above.

Habituated cultures were transferred to a medium containing NAA (1mg/l) and kinetin (0.5mg/l) and non-habituated tissues were transferred to a medium without any additional growth regulators. The effect of growing the two tissue types on media with and without growth regulators, in order to assess growth regulator dependence/ independence, can be seen in Figures 2.1 and 2.2.

The growth rates of both tissue types were dramatically reduced on transfer to the opposite medium type, demonstrating the strong auxin-independent and dependent phenotypes of the habituated and non-habituated tissues respectively. In the non-habituated tissues, growth was completely inhibited after 16 weeks on a medium lacking hormones (Plate 4), whilst the habituated tissue was still able to grow after 16 weeks on the auxin and cytokinin containing medium, but at a dramatically reduced rate (Plate 3).

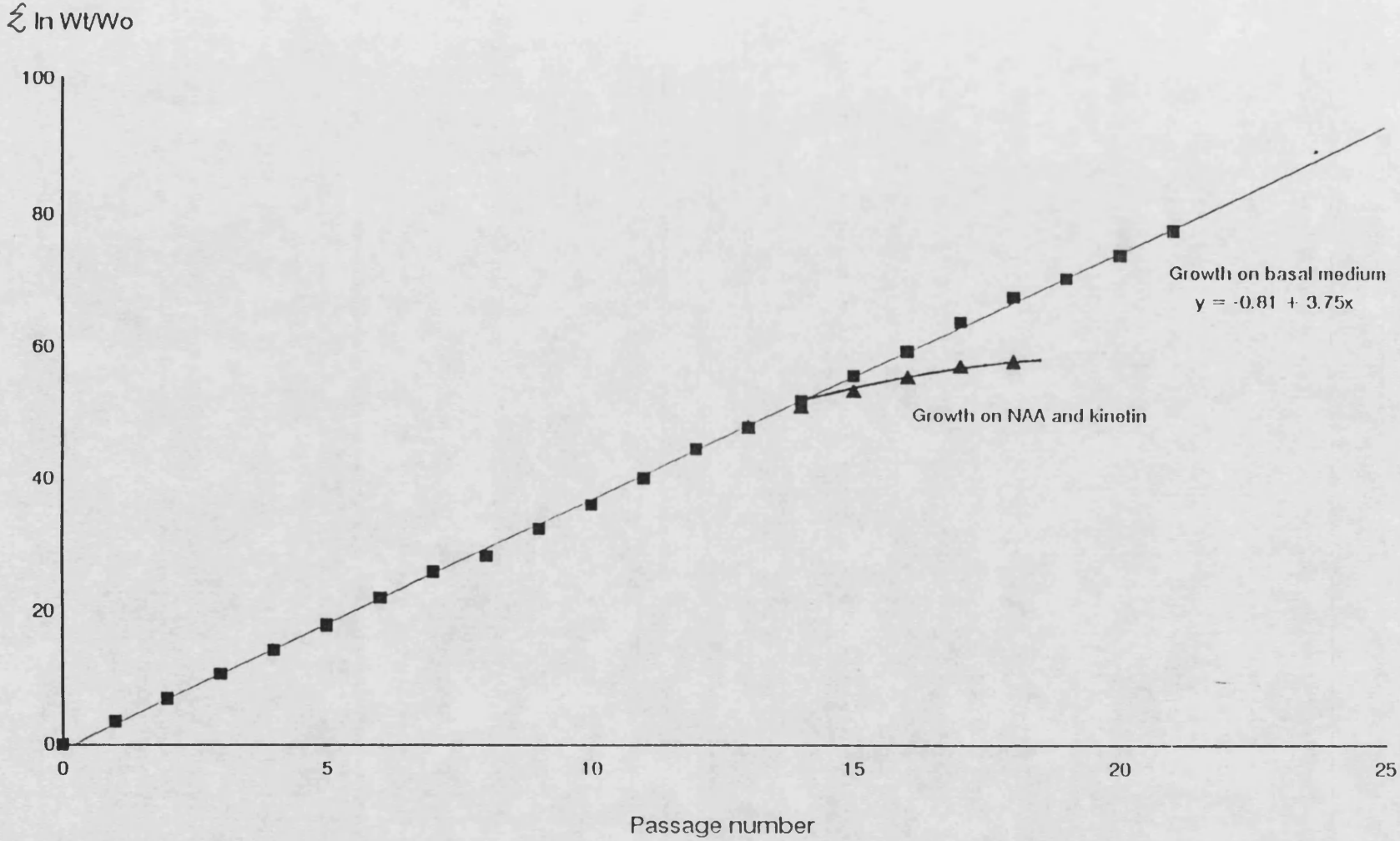


Figure 2.1 : Growth of habituated tissue over 21 three week passages.

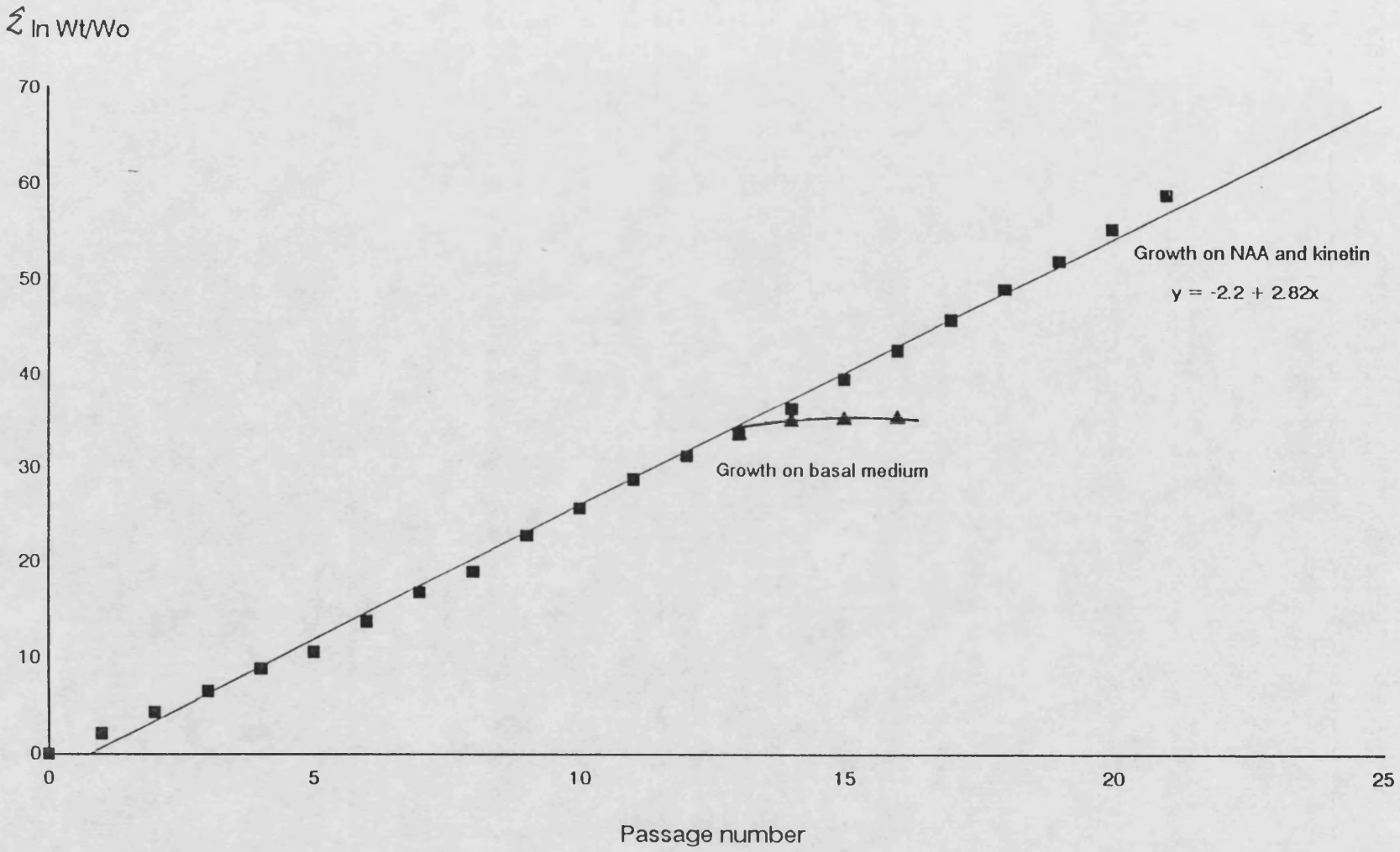


Figure 2.2.: Growth of non-habituated tissue over 21 three week passages.

Plate 1 : Habituated Lactuca callus after 21 days growth on basal medium.

Plate 2 : Non habituated Lactuca callus after 21 days growth on a medium containing NAA (1mg/l) and kinetin (0.5mg/l).

Plate 3 : Comparison of the growth of the habituated tissue on basal medium to that on a medium containing NAA (1mg/l) and kinetin (0.5mg/l).

Plate 4 : Comparison of the growth, after 21 days, of the non habituated tissue on a medium containing NAA (1mg/l) and kinetin (0.5mg/l) to that on basal medium.

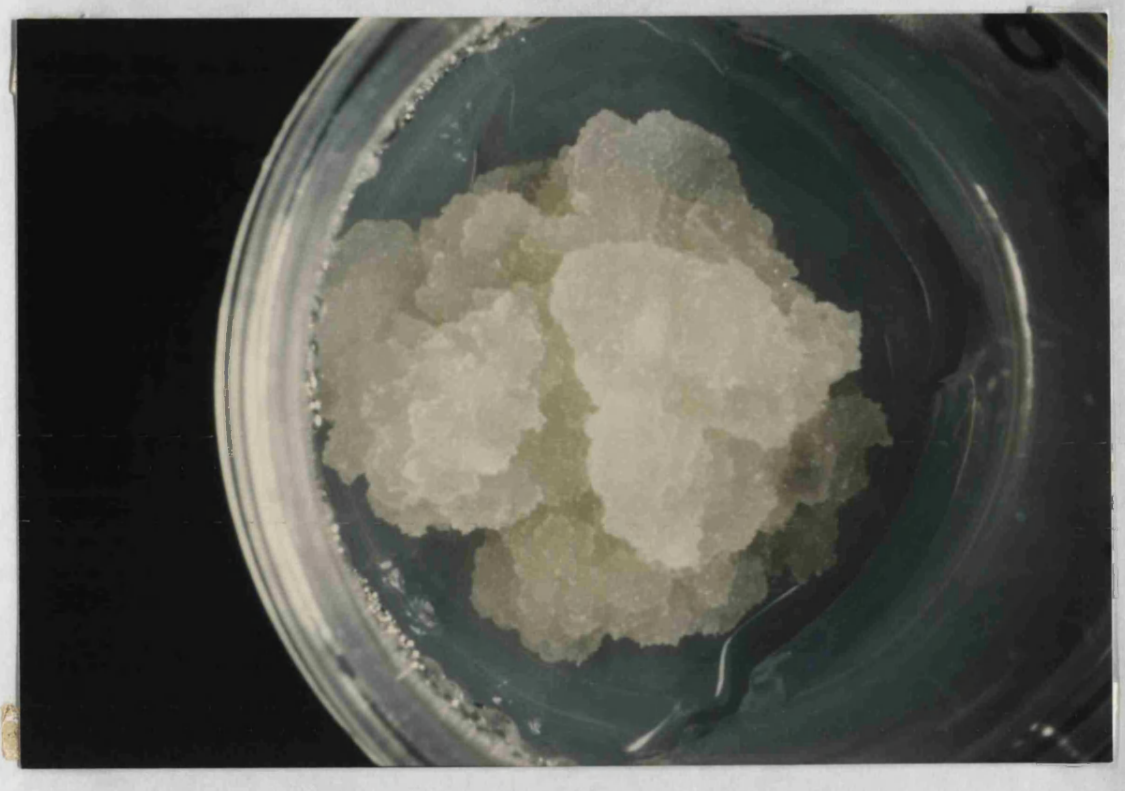


Plate 1 : ⟷ = 1cm

Plate 2 : ⟷ = 1cm

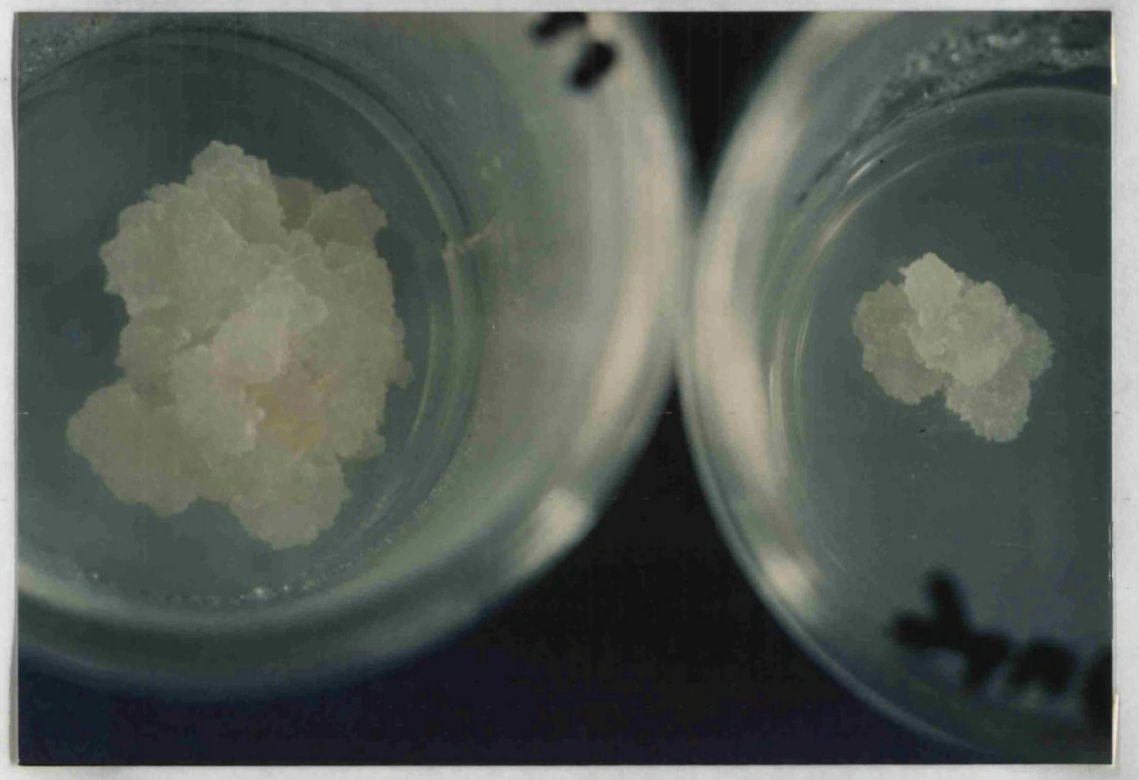


Plate 3 : 1 cm

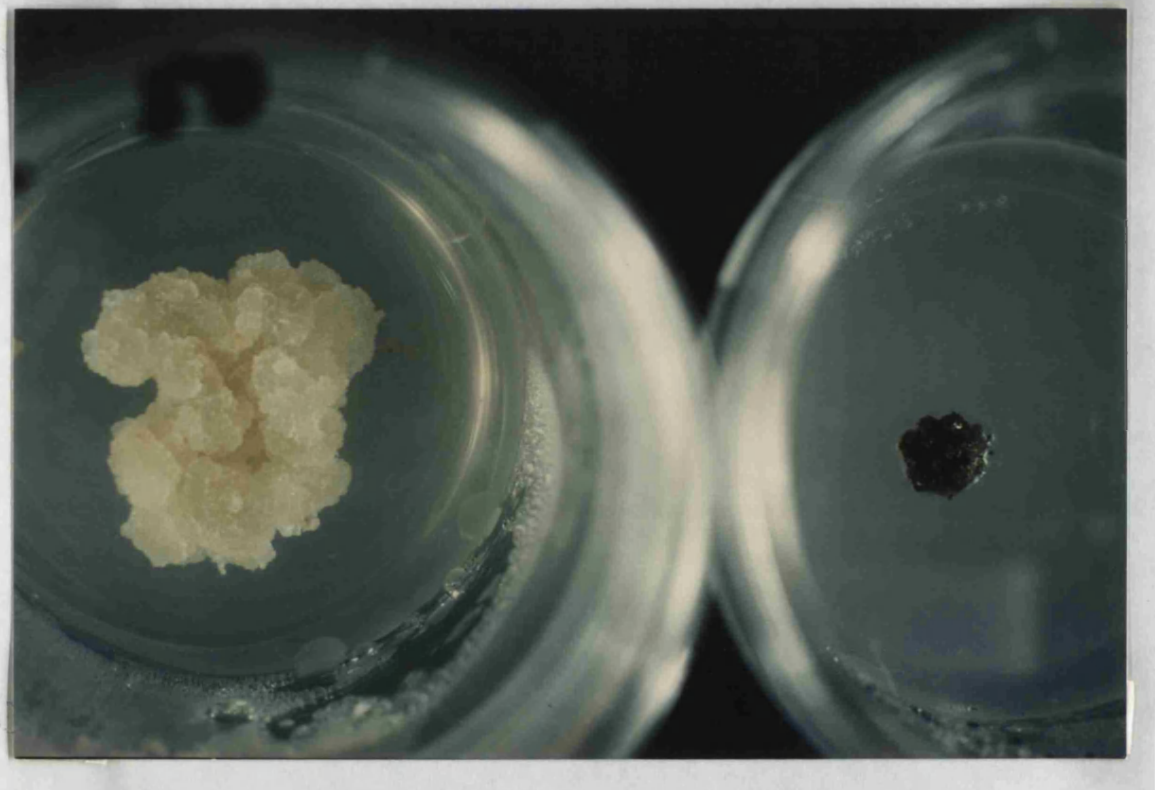


Plate 4 : 1 cm

The habituated and non-habituated tissues were seen to differ in auxin-requirement (Figures 2.1 and 2.2, Plates 3 and 4), pigmentation (Plates 1 and 2), growth rate (Figures 2.1 and 2.2) and expression of organogenic potential (Plate 5).

2.3 Experimental and results

Growth regulator requirements for the induction of callus from Lactuca leaf tissue

Leaf discs of Lactuca sativa (var.L "Lobjoits Green Cos") were isolated and introduced to in vitro culture as described above using media of varying growth regulator content with the aim of determining the requirements for auxin and cytokinin in the induction of callus from this tissue. The cultures were incubated under the standard conditions described above. The auxin and cytokinin content of the media used and the number of discs cultured per treatment are presented in Table 2.1. The concentration of auxin, NAA, used was 1mg/l and of cytokinin, kinetin, 0.5mg/l. No other concentrations of growth regulators were tested. All cultures were incubated at 25°C.

Growth regulator	Length of exposure in days	No. of discs
NAA Kinetin	28 days/continous "	20
NAA	28 days/continous	20
Kinetin	28 days/continous	10
Kinetin NAA	28 days/continous 1 day	10
Kinetin NAA	28 days/continous 2 days	10
Kinetin NAA	28 days/continous 4 days	10

Table 2.1 : Number of leaf discs cultured per treatment. NAA was supplied at 1mg/l and kinetin at 0.5mg/l.

After 28 days in culture, the percentage of leaf discs producing callus on each medium type was recorded and the data are presented in Table 2.2. The callus arising around the periphery of the leaf discs, which was mainly around the cut vein area, was carefully removed and transferred to fresh medium using a sterile scalpel and forceps. The resulting callus cultures were maintained under standard incubation conditions and subcultured every 28 days. At the end of each culture period, the cultures were visually assessed for vigour of growth, appearance and organogenesis prior to transfer to fresh medium. 50mg portions of callus were transferred to the same type of medium and 50mg to a

basal medium (without growth regulators) to determine the percentage of tissues capable of surviving in the absence of growth regulators.

Whenever possible, 10 tissue replicates of each treatment were transferred but some cultures produced insufficient material to allow this and fewer replicates were taken. It was also not always possible to split each replicate for transfer to both types of medium, where there was very little or no growth, but this was done whenever possible. The design of this experiment is presented in Figure 2.3.

Results of callus induction

Callus induction from the leaf tissues was successful on each of the media types tested. The quality and quantity of callus obtained was, however, very much dependent upon the growth regulators present in the culture medium. The most vigourously growing callus arose from leaf discs cultured on a medium containing both NAA and kinetin. Cultures grown on media containing only NAA did not produce very much callus growth and the callus produced was brownish/yellow in colour compared to the pale green tissue initiated on the medium containing both NAA and kinetin. Callus produced in the total absence of exogenous auxin was very brittle in texture and arose in very small quantities. The inability to maintain this culture further *in vitro* suggested that this callus may only have been a response

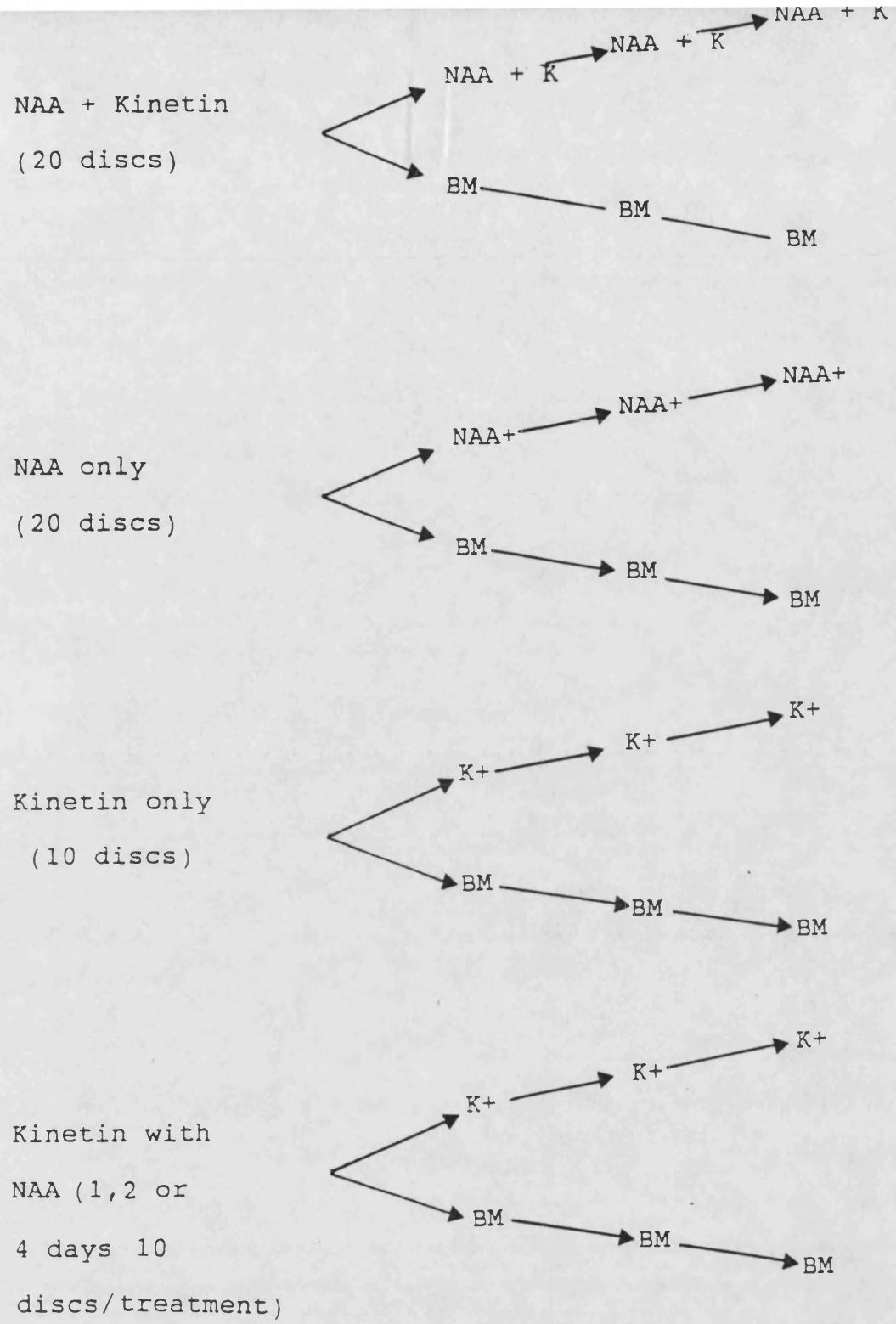


Figure 2.3 : Experimental design of the callus induction experiment.

initiated by wounding of the explant and not influenced by the kinetin present in the growth medium.

An auxin pretreatment of as little as 24 hours made a considerable difference to the quality and quantity of callus produced on media containing only kinetin. The length of the auxin pretreatment (1 to 4 days) did not, however, affect the percentage of explants initially producing callus. The Lactuca tissues appeared to have accumulated sufficient auxin for callus initiation and subsequent growth after only 24 hours.

The ability of an explant to proliferate on a medium lacking growth regulators for a number of weeks does not demonstrate that the tissue is habituated. NAA and kinetin will have been taken up by the tissue from the growth medium or produced endogenously under the influence of these exogenously applied growth regulators. In addition to this, callus tissues consist of a variety of cell types, some of which may act as a form of nurse culture to others in the provision of auxin and cytokinin for growth.

Continuous subculture of callus tissues on a medium lacking growth regulators should, however, eliminate any carry over effect of auxin and cytokinin and select for cell types capable of growing autonomously. After 8/12 weeks in culture on a medium without growth regulators, the possible carry over of auxin and cytokinin from previous culture passages in the presence of growth regulators should be effectively eliminated.

Growth regulator treatment	% of explants producing callus	Passage No. 1	2/3
NAA and kinetin	95% produce healthy callus green/friable	100% of cultures grew	Cultures continue vigourous growth.
NAA only	95% produce yellow/brown callus	80% cultures grew. Vigour variable	New callus arose around unhealthy tissue.
Kinetin	70% produce a response. Very little callus	No further growth	
Kinetin NAA (1 day)	90% produce callus. Growth not vigourous	55% grew Tissue was white and healthy	All NAA pretreated calli grown subsequently on kinetin alone show selection of healthy tissue and the vigour of callus growth and colour of tissue improved.
Kinetin NAA (2 day)	70% produce callus. Quite healthy tissue	50% grew Not very vigourous	
Kinetin NAA (4 day)	90% produce callus. Not very healthy	40% grew Not very vigourous	

Table 2.2 : The percentage of explants producing callus on each of the media tested and the assessment of appearance and vigour of growth of the callus produced.

GROWTH REGULATOR TREATMENT	SURVIVAL AND GROWTH ON TRANSFER TO BASAL MEDIUM
NAA and kinetin	Initially, 90% of cultures grew with variable vigour. 40% grew healthily and 50% gave very little growth or produced nodules of growth on underlying senescent tissue. 15% of the cultures produced shoots on initial transfer to basal medium but this tissue was rapidly overgrown by disorganised callus on further culture on this medium. Habituated tissues were isolated.
NAA only	100% of cultures begin to senesce on initial transfer but 70% of these produced proliferating growth on the surface of these cultures. Habituated tissues were isolated.
Kinetin only	Insufficient callus was initiated on the leaf discs to provide more than 5 replicates. All cultures died on transfer to basal medium.
Kinetin with 1, 2 or 4 day NAA treatment	All of these cultures respond in the same way to culture on basal medium. After 2/3 weeks 80% (1 day), 50% (2 day) and 50% (4 day) of the cultures produce proliferating nodules on the surface of senescent tissue. Habituated tissues were isolated.

Table 2.3 : The growth of callus cultures on transfer to a medium without growth regulators.

The response of each of the different callus types to culture on a medium free of growth regulators, after an initial 28 day culture period on media containing NAA and/or cytokinin, can be seen in Table 2.3. Cultures capable of growing on a medium free of additional growth

regulators for a period of upto 12 weeks were obtained from calli initiated on all media types, with the exeption of those grown in the complete absence of auxin. In these cultures, insufficient callus tissue was obtained for the transfer of healthy tissue to a medium without growth regulators and those tissues which were transferred died. The initial lack of response in the initiation of callus tissues from leaf discs grown in the absence of additional auxin was one reason for the inability to isolate habituated tissues from these cultures.

Habituated tissues generally arose as small, pale, friable portions of tissue growing on the surface or edge of brown senescent tissue which did not grow initially on transfer to a medium without auxin or cytokinin (see Plate 6). These areas of growth may have arisen as a result of growth substances being released from the underlying senescent tissue and this might explain why only a proportion of these tissues on subsequent transfer to basal medium were apparently habituated (Tables 2.4, 2.5, and 2.6). Some of the tissues were, however, capable of further growth on media without auxin and cytokinin and were demonstrated as habituated, by continuing to grow on basal medium for up to 16 weeks after transfer from media containing growth regulators.

Calli initiated on a medium containing both NAA and kinetin provided the best tissue from which to isolate habituated cultures. After 12 weeks in culture on basal

Plate 5 : Roots produced on the non habituated tissue after 28 days growth on a medium containing NAA (1mg/l) and kinetin (0.5mg/l).

Plate 6 : New callus growth on the surface of non habituated tissue cultured on basal medium for 21 days. A proportion of these new callus growths were shown to be habituated.

Plate 5 : ⟷ = 0·1cm

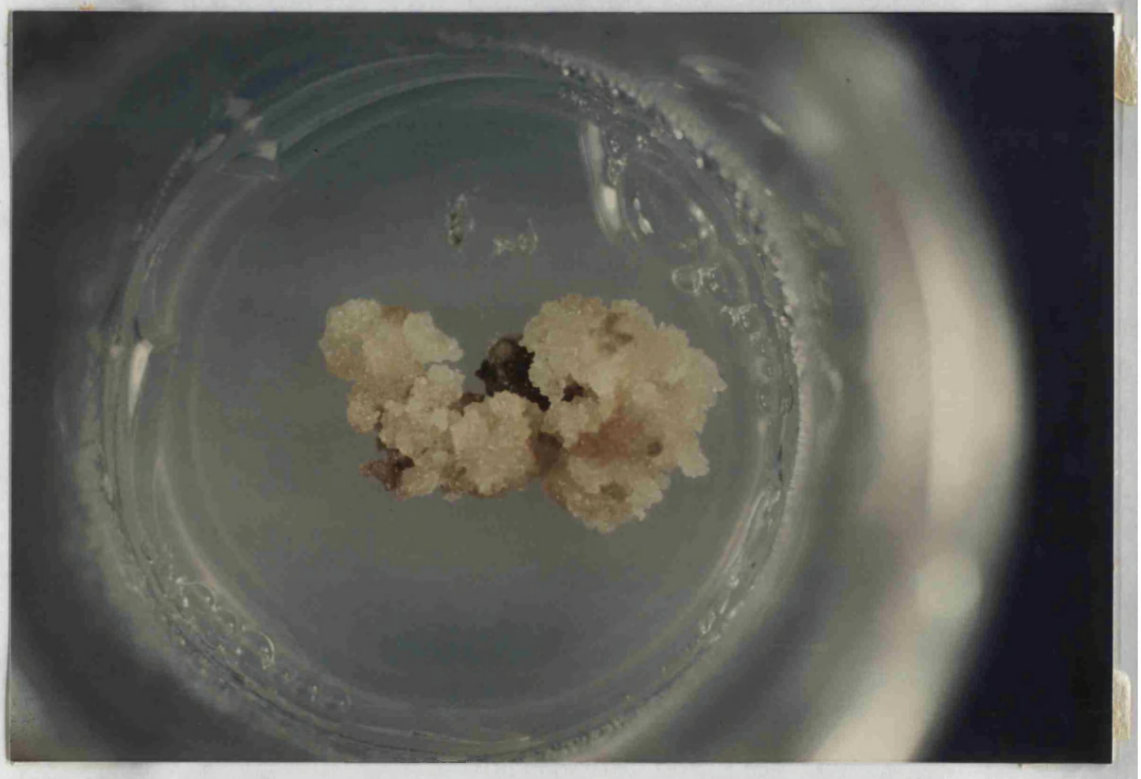


Plate 6 : ⟷ 1cm

medium, 90% of these cultures still grew, 30% of these quite vigorously (Table 2.4). The difference in the vigour of growth of callus tissues, initiated on different induction media, on transfer to basal medium was probably the result of a difference in the quality of callus tissue initially isolated. Calli initiated on a medium containing both auxin and cytokinin were the most vigorously growing tissues produced and this vigour appeared to have influenced the subsequent ability of these tissues to grow basal medium.

Period of growth on basal medium (weeks)	Percentage and vigour of growth of tissues on basal medium		
	+	++	+++
4	10	50	40
8	20	60	20
12	10	60	30

Table 2.4: The percentage and vigour of growth of cultures initiated on a medium containing both NAA and kinetin (28 day passage) on transfer to basal medium.

Period of growth on basal medium	Percentage and vigour of growth of tissues on basal medium		
	+	++	+
4	30	70	0
8	50	50	0
12	70	30	0
16	80	20	0

Table 2.5: The percentage and vigour of growth of cultures initiated on a medium containing only NAA (28 day period) on transfer to basal medium.

Period of growth on basal medium	Percentage and vigour of growth of tissues on basal medium		
	+	++	+++
4	20	80	0
8	60	30	10
12	70	20	10

Table 2.6: The percentage and vigour of growth of cultures initiated on a medium containing only kinetin (28 days, with a 24 hour NAA pretreatment) on transfer to basal medium.

Legend for tables 2.4, 2.5 and 2.6. :

\+ No growth ++ Some growth, including growth on senescent tissue (eg. Plate 6).

+++ More vigourous growth (> 1 cm diameter).

Organogenesis

Overall, the level of organogenesis was low in this series of experiments within the subculture passages of 28 days on media with and without growth regulators. The tissues isolated on media containing both NAA and kinetin demonstrated a low degree of shoot production. Approximately 15% of the cultures produced shoots, the first appearing on a callus transferred directly from leaf disc to basal medium. These shoots appeared after approximately 5 weeks in culture. On transfer to basal medium, any organogenic tissue in the cultures was overgrown with disorganised callus. Cultures maintained on media containing both NAA and kinetin produced many shoots when left for longer than 28 days without subculture to fresh medium.

Roots were initiated directly from 10% of the leaf discs grown on medium containing only NAA. Overall, root production was low in callus tissues maintained on NAA containing medium during the culture passage of 3 weeks,

with only 20% of the cultures responding, but roots were produced by the majority of these tissues when left longer than 28 days on this medium. As with shoot production, the transfer of organogenic tissues to basal media resulted in the organised areas being covered with disorganised growth. The root producing cultures (grown on media containing only NAA) produced very little callus tissue.

2.4 Discussion

The results of the callus initiation experiment in Lactuca sativa var.L leaf tissue indicated that auxin was an absolute requirement for the induction of callus from this tissue. The callus initiated from leaf discs cultured on a medium without any additional auxin was apparently only a wound response, as this tissue could not be maintained on transfer to fresh medium.

In vitro grown plant tissues exhibit a wide variety in their requirement for growth regulators with respect to the induction of callus. The explant response is dependent upon species (Bayliss and Dunn 1979), the developmental state of the explant and the endogenous levels of growth regulators (Yeoman and Forche 1980). The role of auxin appears most crucial to callus induction as many tissue explants are capable of dedifferentiating in the absence of exogenously applied cytokinin, including Jerusalem artichoke (Yeoman and Mitchell 1970), walnut (Rodriguez 1982) and ginseng (Chu et al 1978). Very few cultured explants, however, initiate callus in the complete absence of exogenously

applied auxin. Noteable exeptions are the citrus fruit sacs cultured by Kordan (1959).

Auxin-habituated tissues were derived from calli induced on all media types with the exeption of those initiated in the complete absence of auxin. Insufficient callus was produced on this medium for transfer to basal medium. The Lactuca sativa var.L leaf explant tissue appeared to habituate quite readily. This was shown by the ease with which cultures were obtained which were capable of growing on basal medium after only a short period in culture (28 days) in the presence of additional growth regulators. The readiness of certain species to habituate after only short periods in culture has been documented (eg. Buiatti et al 1970, Bennici 1972, Sheridan 1968). Buiatti et al (1970) reported the ability of in vitro grown tissues of tobacco to grow on auxin free medium after only short term exposures to 2,4-D of 7 and 20 days. Increasing the concentration of 2,4-D in the pretreatment was found to increase the percentage of cultures which became habituated for auxin.

In this present work, only a proportion of the Lactuca calli, initially able to grow on basal medium (after 4/8 weeks), were able to proliferate on subsequent transfers to media without additional growth regulators (Tables 2.4, 2.5, and 2.6). This suggested that many of the tissues were able to grow initially because of the carry over of auxin and/or cytokinin from the previous culture passage. Bender and Neumann (1978) have shown that a short auxin

pretreatment of 6 days was sufficient to maintain the growth of carrot cultures for a period of 28 days without auxin. The 28 day auxin and/or cytokinin treatments used in this present experiment could feasibly maintain auxin and cytokinin-requiring tissues for longer than a single 4 week passage.

The demonstration that a culture is habituated requires more than an observation that the tissues are able to grow in the absence of growth regulators for a single culture passage. The degree of habituation, R, may be determined in order to assess the degree of habituation for growth regulators in a given tissue. This requires the analysis of growth over a number of sucessive culture passages (Meins and Binns 1977).

Overall, callus cultures derived from Lactuca sativa var.L leaf tissue may be considered as capable of habituating quite readily given adequate auxin pretreatment for the induction of healthy callus tissue. Tissues initiated in the presence of both NAA and kinetin were the healthiest and most vigourously growing calli produced and gave rise to the highest proportion of tissues capable of proliferating on basal medium for more than 12 weeks (Table 2.4). Tissues capable of growth on basal medium for this length of time were considered to be habituated as the effect of carryover of growth regulators from previous incubations could effectively be ignored. Cultures initiated in the presence of NAA alone also produced some habituated tissues on transfer to basal medium (20% of

cultures transferred), as did those tissues initiated and grown on media containing only kinetin, with only a 24 hour auxin pretreatment (30% of cultures transferred).

No vigourously growing habituated tissues were isolated from cultures initiated in the total absence of applied cytokinin whereas 30% and 10% of transferred cultures, initiated in the presence of cytokinin with auxin treatments of 28 and 1 day respectively, grew vigourously.

To determine a more direct role of auxin and cytokinin in the induction of habituation is extremely difficult as this require the exposure of healthy callus, which requires auxin and cytokinin for growth, to various treatments with these growth regulators. It is important that the calli used are shown to require growth regulators for proliferation because partially habituated cultures, for example, if the chosen tissue habituated readily, would interfere with the determination of growth regulator involvement.

Chapter 3

Analysis of endogenous IAA in habituated and non-habituated Lactuca callus tissues

3.1 Introduction

In order to examine the role of endogenous IAA in the initiation and maintenance of auxin-habituation, it is necessary to identify IAA in habituated tissues and quantify the levels present. This Chapter describes the analysis of IAA from small amounts of Lactuca callus tissues using the physicochemical techniques of HPLC and GC-MS. The levels of IAA in habituated tissues were compared to those in a non-habituated culture, with the tissues analysed at seven day intervals throughout a twenty-one day culture period.

The extent of purification required for the analysis of IAA from plant material is ultimately dependent upon the complexity of the tissue to be analysed. The Lactuca callus tissues were relatively simple (disorganised) and low in pigmentation. Rigorous, time-consuming purification steps were therefore avoided. Mini-chromatography columns such as Sep-Pak cartridges, which are commercially available, are now in widespread use for the purification of plant growth regulators including IAA (Sandberg et al 1987, Akiyama et al 1983, Law and Hamilton 1982). These Sep-Pak cartridges were employed in this present work in the purification of IAA from Lactuca callus tissues.

IAA was quantified initially by HPLC using

fluorescence for detection in preference to the alternative U.V. detector as U.V. absorbance is less specific for the detection of IAA (Akiyama etal 1983). HPLC-F has been employed sucessfully for the estimation of IAA levels in plant tissues after only minimal pre-purification (Akiyama et al 1983, Law and Hamilton 1982).

GC-MS analysis of plant extracts was also performed in an attempt to confirm the presence of IAA in the tissues and to validate the HPLC-F technique for the quantification of endogenous IAA. This instrument became available in the final few months of this work and therefore this analysis was limited. Losses of IAA during purification were monitored by the introduction of 5-^{3}(H)IAA and d_2IAA as internal standards, for HPLC-F and GC-MS analysis respectively, at the extraction stage of the purification procedure.

3.2 Materials and methods

Plant material

The auxin-habituated and non-habituated callus tissues used for auxin analysis were subcultured from stock cultures, isolated and maintained as described in Chapter Two. Explants (50mg) of callus tissue were transferred to 100ml plastic petri jars (Sterilin Ltd.) with transparent lids which contained 25ml of Murashige and Skoog medium (pH 5.5, 2% sucrose, 0.7 % agar, Murashige and Skoog 1962). The medium for the non-habituated cultures was supplemented with NAA (1mg/l) and kinetin (0.5mg/l). The cultures were

grown under the standard conditions described in Chapter Two for periods of 7, 14 or 21 days. At each of these time periods, three replicate samples (2g fresh weight) of each callus type were harvested and placed into 5 x 1cm plastic storage vials (Sterilin Ltd). The tissues were then immersed in liquid nitrogen and stored at -196°C in a liquid nitrogen storage vessel (B.O.C. London.) prior to analysis.

Chemicals

All chemicals were obtained from Sigma Chemical Company with the exeption of deuterium oxide and sodium deuteroxide which were purchased from Aldrich Chemical Company Inc. HPLC grade solvents and acetic acid were obtained from Rathburn Chemicals Ltd. Walkerburn, Scotland. These HPLC grade reagents were filtered and degassed prior to use in HPLC.

All other solvents were of analytical grade with the exeption of diethyl ether, which was Aristar grade, and were purchased from B.D.H. Poole, England.

5-^{3}(H)-indolylacetic acid (925 GBq/mmol) was purchased from Amersham International plc. and Optiphase "Safe" scintillant was obtained from LKB. C18 and silica Sep-Pak cartridges were purchased from Waters Associates, Massachusetts, USA.

All glassware used in auxin analysis was first soaked in concentrated sulphuric acid following a preliminary wash in a detergent solution. The acid was removed by rinsing in

tap water followed by three rinses in distilled water. The glassware was then oven-dried at 40-50°C.

Extraction and purification of IAA

2g (fresh weight) of callus tissue (still frozen) was added to 10ml of extractant (80% aqueous methanol containing 10mM sodium diethyldithiocarbamic acid) in a 50ml glass homogeniser tube. This concentration of anti-oxidant was added to all of the solvents used throughout the purification procedure. Approximately 2.5 x 10^6 dpms of 5-^{3}(H)IAA was added to the solution as an internal standard and the contents of the tube were then homogenised with a teflon plunger. A further 10ml of extractant was added to provide a final w:v ratio of tissue:extractant of 1:10 . The homogenate was left on ice in dim light for 45 minutes before filtration through a Whatman No.1 filter under suction. This filtrate was stored at 4°C while the remaining tissue was re-extracted in a further 20ml of the extractant. After 45 minutes this extract was also filtered under suction as before.

The tissue extracts were pooled, then reduced to the aqueous phase in a 250ml flask at 40°C in vacuo. This stage was observed when the rate of evaporation decreased and the extract became viscous. The aqueous extract (approximate volume 8ml) was taken up in 0.1M phosphate buffer (pH 8) and transferred to a 40ml glass stoppered tube providing a final volume of approximately 15ml. The extract was then partitioned twice against an equal volume of ethyl acetate.

The organic phases were discarded and the remaining aqueous phase acidified to pH 3 by the addition of 2M HCl. The acidic extract was partitioned twice against equal volumes (approx. 15ml) of ethyl acetate. The organic phases (containing acidic indoles (Fig.3.1)) were pooled and evaporated to almost dryness in a 250ml flask at 40°C *in vacuo*. The extract was then taken up in 5ml of 20mM sodium acetate buffer (pH 2.7) and loaded onto a C18 Sep-Pak cartridge preconditioned with this buffer.

The loaded cartridge was rinsed with 2 x 5ml volumes of the buffer prior to elution of the extract with 5ml of a solution of 1% acetic acid in hexane:ethyl acetate (9:1, w:v) onto a silica Sep-Pak cartridge preconditioned with 0.5M acetic acid. The loaded cartridge was then rinsed with 2 x 5ml volumes of the hexane:ethyl acetate solution (9:1, w:v) before elution of the extract with 5ml of 1% acetic acid in hexane:ethyl acetate (2:3, w:v) into a 5cm glass centrifuge tube. The eluant was evaporated to dryness by centrifugal freeze drying in a Savant speed vacuum concentrator fitted to an Edwards freeze dryer. The extract was then taken up in 0.5ml of HPLC eluant (1mM acetic acid in 45% aqueous methanol), passed through a 0.2μ filter (Gelman Sciences) and stored in a glass vial at -20°C prior to HPLC analysis.

List of abbreviations for Figure 3.1

IEt	indole-3-ethanol
IM	indole-3-methanol
IAld	indole-3-aldehyde
IAA	indole-3-acetic acid
5-OH-IAA	5-hydroxyindole-3-acetic acid
4-Cl-IAA	4-chloroindole-3-acetic acid
ICA	indole-3-carboxylic acid
OxIAA	oxindole-3-acetic acid
IAAsp	indole-3-acetylaspartic acid
IAGlu	indole-3-acetylglutamic acid
IPyA	indole-3-pyruvic acid
PAA	phenyl acetic acid
Trp	tryptophan
IAGluc	1-O-indole-3-acetyl-β-D-glucose
IAInos	indole-3-acetyl-myoinositol

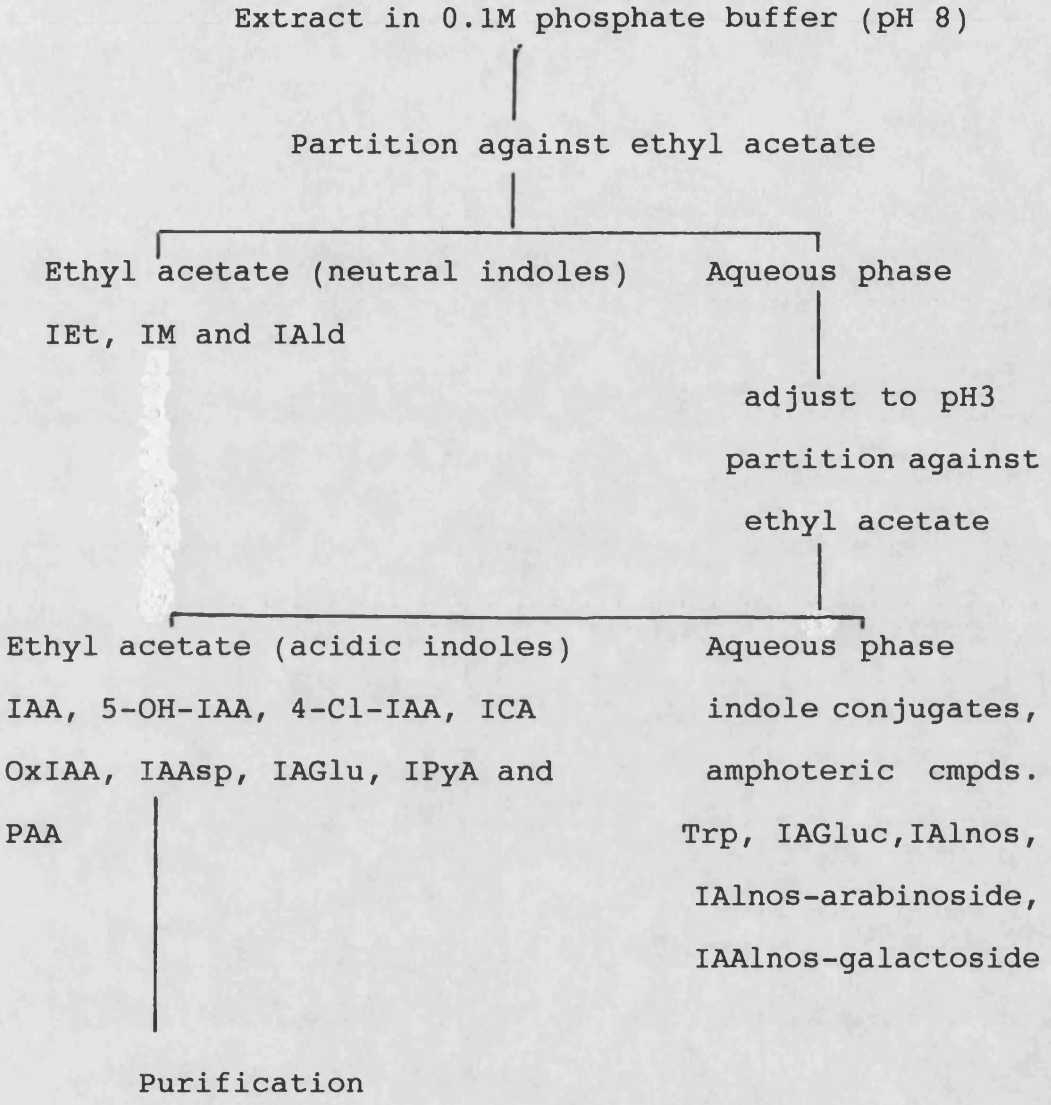


Figure 3.1 : Solvent partitioning scheme used for the purification of acidic indoles (Sandberg et al 1987).

The determination of loss during purification

The individual purification steps in the method developed for the analysis of IAA in Lactuca callus tissues were assessed for their contribution to the overall loss of IAA which occurred during purification. This was achieved by adding a standard amount of IAA (0.7μM), made up in the appropriate solutions used for each purification step, to each of the purification steps and analysing the amount of IAA recovered on HPLC. Ideally, a radiolabelled IAA standard should be employed for this type of analysis, allowing the addition of plant material for a more meaningful analysis. The 5-^{3}(H)IAA available was not, however, considered of sufficient purity for use in this experiment (see pages 98 to 101).

The samples recovered from each purification step were evaporated to dryness and then taken up in 0.5ml of HPLC solvent (1mM acetic acid in 45% methanol). 20μl aliquots of the recovered samples were loaded onto a Spherisorb ODS 1 (250 x 4.6mm) analytical HPLC column. The IAA was eluted under the standard HPLC conditions described for this analytical column and the fluorescent peaks of IAA recovered from each step were integrated by a CI-10B integrator (LDC/Milton Roy). These areas were compared to the area produced by a standard quantity of IAA (0.7μM) in order to determine the losses which had occurred during each step (Appendix B). Losses measured for the final step of the purification (step 7), which involved the evaporation of the sample by centrifugal freeze drying,

were subtracted from those monitored at each purification step as this procedure was common to all. The percentage contribution of each step to the overall loss of standard IAA was then calculated (Figure 3.2).

Purification steps

(1) Extraction

10ml of a 7μM methanolic solution of IAA was added to 30ml of the extractant used in the purification (ie.80% aqueous methanol containing 10mM of sodium diethyldithiocarbamic acid) in a 50ml glass homogeniser tube. A teflon plunger was then used to introduce air into the sample. The solution was then left on ice in dim light for a total of 90 minutes. No plant material was added as in the absence of radiolabelled IAA no distinction could be made between standard and plant derived IAA. The solution was then filtered through a Whatman No.1 filter under suction. A 4ml volume of the filtrate (total volume of 40ml) was removed and evaporated to dryness by centrifugal freeze drying. This sample represented 1/10th of the original extract ie.0.7μM.

(2) Rotary film evaporation of the crude extract

10ml of a 0.7μM IAA solution dissolved in extractant was evaporated to the aqueous phase in a 250ml flask at 40°C *in vacuo* by rotary film evaporation. The 2ml of aqueous solution recovered was removed by pasteur pipette and the flask then rinsed with 3ml of 0.1M phosphate buffer

(pH 8). The washings of the flask were added to the 2ml of extract and the 5ml solution then evaporated as above.

(3) Solvent partitioning

5ml of a 0.7μM IAA solution in 0.1M phosphate buffer (pH 8) was partitioned twice against 5ml volumes of ethyl acetate containing the anti-oxidant (10mM). The organic phase was removed in each case and discarded. The IAA solution was then acidified to pH 2.7 by the addition of 2M HCl and then partitioned against 5ml of ethyl acetate. The organic phase was carefully removed by pasteur pipette and transferred to a 5ml glass centrifuge in which the sample was evaporated to dryness as above.

(4)Rotary film evaporation of ethyl acetate

10ml of a 0.7μM solution of IAA in ethyl acetate was evaporated to almost dryness in a 250ml flask at 40°C *in vacuo* by rotary film evaporation. The IAA was then taken up in 5ml of 20mM sodium acetate buffer (pH 2.7), prior to evaporation in the vacuum concentrator.

(5)C18 Sep-Pak

5ml of 0.7μM IAA in 20mM sodium acetate buffer (pH 2.7) was loaded onto a C18 Sep-Pak cartridge preconditioned by the following; 2ml of methanol followed by 2ml of distilled water and finally 5ml of the sodium acetate mobile phase (pH 2.7). The loaded cartridge was then washed with 2 x 5ml volumes of this buffer and the IAA then eluted

with 5ml of 1% acetic acid in hexane:ethyl acetate solution (9:1,v:v) into a centrifuge tube in which the solution was evaporated to dryness.

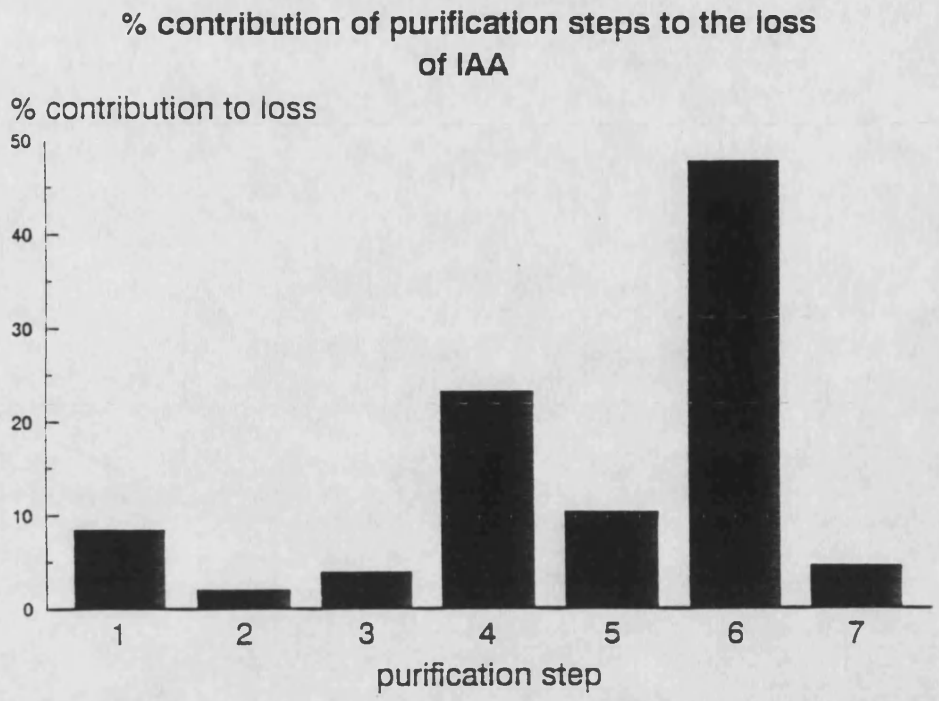
(6) Silica Sep-Pak

5ml of 0.7μM IAA in 1% acetic acid in hexane/ethyl acetate (9:1,v:v) was loaded onto a silica Sep-Pak cartridge preconditioned with 5ml of 0.5M acetic acid. The cartridge was then washed with 2 x 5ml volumes of this solution and the IAA eluted in 5ml of 1% acetic acid in hexane:ethyl acetate solution (2:3,v:v) into a centrifuge tube for freeze drying.

(7) Centrifugal freeze drying

5ml of 0.7μM IAA in 1% acetic acid in hexane:ethyl acetate solution (2:3,v:v) was evaporated to dryness in a centrifugal freeze dryer. All solutions recovered from steps 1-6 were evaporated to dryness in this way. All of the above samples were taken up in 0.5ml of HPLC eluant for subsequent HPLC analysis to determine the recovery of IAA from each step.

The results presented in Figure 3.2 demonstrate that losses of IAA were incurred at all stages throughout the purification procedure. The most significant losses were, however, introduced by the Sep-Pak cartridges. Budini et al (1982) experienced problems in the use of Sep-Pak cartridges for the purification of IAA from immature grape tissues. These authors reported that IAA passed through a



1. Extraction
2. Rotary evaporation of crude methanolic extract
3. Solvent partitioning against ethyl acetate
4. Rotary evaporation of ethyl acetate fraction
5. C18 Sep-Pak
6. Silica Sep-Pak
7. centrifugal freeze drying

Figure 3.2 : The percentage contribution of each purification step to the overall loss of IAA.

C18 Sep-Pak column without binding to the stationary phase. The binding of IAA to C18 reverse phase columns is very much a pH dependent process and Akiyama et al (1983) reported no difficulties in binding IAA to these cartridges if the pH was maintained at 3.5 or below. The C18 and silica Sep-Pak cartridges used in this work were maintained below pH 3.5 by sodium acetate buffer (pH 2.7) or 0.5M acetic acid.

An alternative explanation for the poor recoveries of IAA from these cartridges in this work may have been that the IAA was too strongly bound to the column to be removed by 5ml volumes of the hexane:ethyl acetate solutions employed as the eluant. Such a problem was not, however, identified in preliminary investigations of the retention characteristics of these cartridges where recoveries of over 70% IAA were obtained. As these cartridges were obtained from a commercial source, it is inevitable that batch to batch variations do exist which could influence the chromatographic properties of these mini-columns (Sandberg et al 1987). Each batch of cartridges should, ideally, be tested for the adequate recovery of IAA by the chosen mobile phase.

Plant tissues release autolytic enzymes on maceration (Yokota et al 1980) and this would increase losses incurred at stage (1). Homogenisation is not, however, always necessary for the extraction of auxin from callus tissues (Horemans et al 1984).

Analytical HPLC

Standard IAA and semi-purified plant extracts were loaded in 20µl aliquots onto a Spherisorb ODS analytical HPLC column (250 x 4.6mm, LDC/Milton Roy) via a Rheodyne injection valve fitted with a 20µl injection loop. A flow rate of 1ml/min was provided by two pumps (Constametric III metering, LDC/Milton Roy). Fluorescence in the samples was detected by an LDC/Milton Roy fluoromonitor (Model 1311) fitted with a Hg lamp with excitation and emission set at 254mn and 355nm respectively. Under these conditions, standard IAA eluted at approximately 6.5-7.5 minutes in an isocratic solvent system of 1mM acetic acid in 45% methanol.

Retention times for standard IAA were observed to decrease with increases in room temperature in the absence of an insulating thermal jacket for the column. This required frequent injections of an IAA standard between samples to confirm the retention time of authentic IAA. The fluorescent peaks produced on the chromatograms were automatically integrated by an LDC/Milton Roy integrator (CI-10B). A linear relationship between peak area and the amount of IAA injected was found to be reproducible (eg.Fig. 3.3) despite variations in the peak areas produced for the same quantity of IAA on different days of analysis.

Preparative HPLC

The identification and quantification of IAA by GC-MS requires a greater quantity of IAA than HPLC analysis. Up

to 200ng of IAA may be required for full scan mass spectrometry (Sandberg et al 1987). The quantity of callus tissue taken for extraction was therefore increased from 2g to 12-20ng (fresh weight) for GC-MS analysis. The crude callus extracts were purified by splitting the initial extract into smaller volumes and analysing these fractions by the purification technique described above. The pooled extract of these fractions were then subjected to a preparative HPLC separation on a Spherisorb S5 ODS1 HPLC column (250 x 10mm i.d., Anachem, Luton, Beds.) in place of the analytical HPLC step employed for the smaller extracts.

Semi-purified callus extracts were loaded onto the larger column via a Rheodyne injection valve fitted with a 100µl injection loop. A flow rate of 4ml/min gave a retention time of approximately 7-8 minutes for standard IAA. Samples were eluted isocratically in 1mM acetic acid in 45% methanol as before. Fluorescent peak areas were determined as described above. A linear relationship between peak area and the amount of IAA injected was reproducible on this column (Fig.3.4).

Preparation of extracts for GC-MS analysis

Putative IAA peaks on HPLC profiles with retention times close to or co-incident with that of standard IAA were collected, manually, in 40ml glass tubes wrapped in aluminium foil and stored on ice. Peaks with retention times identical to that of IAA were collected from the

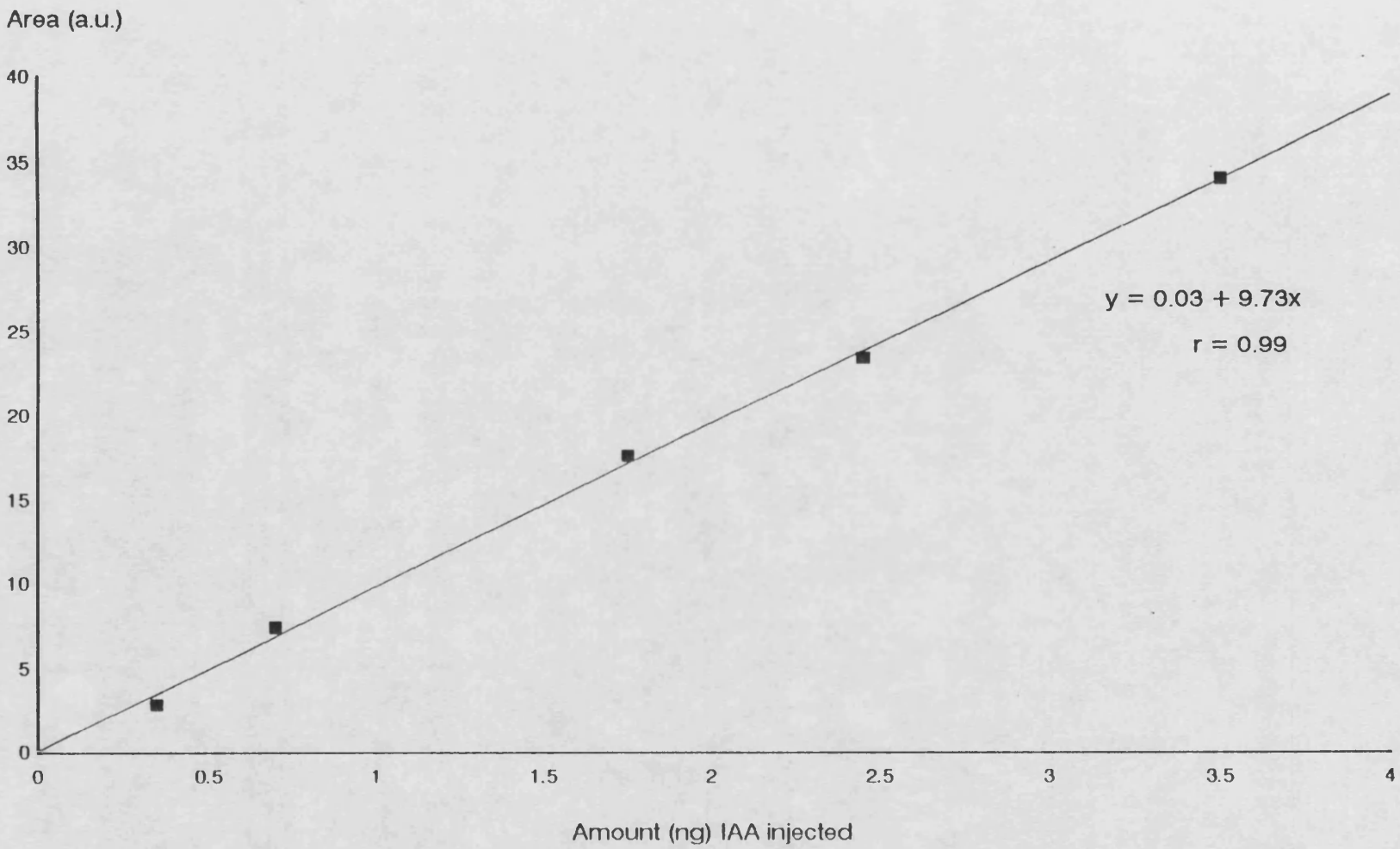


Figure 3.3 : Standard IAA curve on an ODS analytical HPLC column.

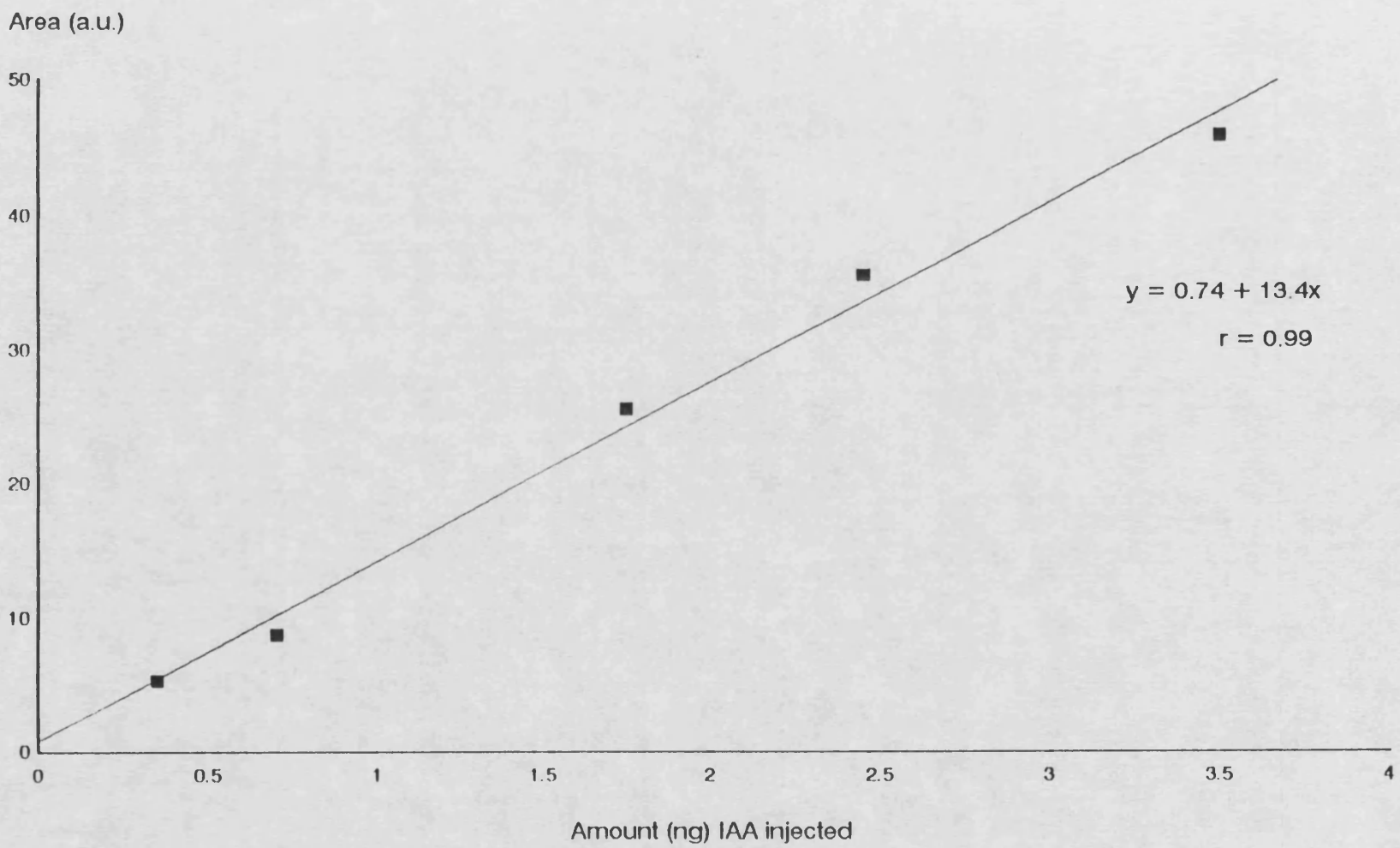


Figure 3.4 : Standard IAA curve on an ODS preparative HPLC column.

entire callus extract in this way and then redistributed in 5ml volumes to a series of small glass centrifuge tubes. The samples were evaporated to dryness in a centrifugal freeze dryer. The extract in each tube was then taken up in approximately 0.5ml of methanol and transferred to a single 5ml centrifuge tube.

The pooled extract was evaporated to dryness by centrifugal freeze drying before redissolving in 1ml of diethyl ether. The sample was then transferred to a 3ml glass reactivial (Pierce Chemical Co.) and dried under a stream of nitrogen. This process of transferring residue of the sample from the centrifuge tube to the reactivial was repeated. The sides of the vial were then washed with several small volumes of diethyl ether which were then evaporated. This process of washing down residue from the sides of the vial concentrated the sample into the conical portion at the base of the vial in preparation for derivatisation.

The production of diazomethane

The volatility of IAA was increased for GC-MS analysis by derivatisation. Diazomethane is very effective for the methylation of carboxy acids such as IAA (Cohen 1984). The apparatus used for the generation of diazomethane was made by the glassblower at the University and was based on the design of Cohen (1984). This equipment is illustrated in Figure 3.5. All parts were of borosilicate glass.

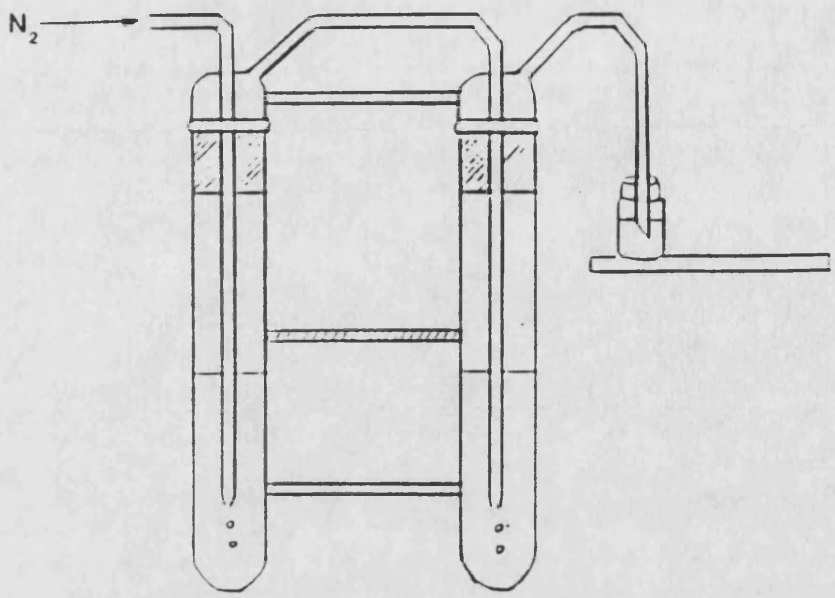


Figure 3.5 : The apparatus used for the generation of diazomethane.

Tube 1 was half-filled with approximately 25ml of diethyl ether. A mixture of diethyl ether (7ml), 2-(2ethoxyethoxy) ethanol (7ml) and 7M sodium hydroxide (7ml) was added to tube 2 and the contents thoroughly mixed using a glass rod. A flow rate of nitrogen was set at approximately 5-10 ml/min through both tubes. 1g of N-methyl-N-nitroso-p-toluene sulphonamide was added to tube 2 via a small glass funnel assisted by a small volume of diethyl ether. The apparatus was then sealed with metal springs and the PTFE outlet from Tube 2 placed directly into a 3ml glass reactivial containing 2ml of diethyl ether.

After a few minutes, the ether in the vial developed

the characteristic bright yellow colouration of ethereal diazomethane and the vial was removed, sealed and replaced with a fresh vial containing diethyl ether. The process continued with the collection of gas in ether until a full colour did not develop in the ether. The reaction was halted by the removal of the nitrogen flow and the addition of excess 10% acetic acid in ether to Tube 2. The entire procedure was undertaken in a fully ventilated fume hood because of the toxic nature of diazomethane.

Methylation of IAA by diazomethane

Standard IAA and peaks recovered from plant extracts with co-incident retention times with IAA were methylated prior to GC-MS analysis by the method described by Cohen (1984). 1ml of ethereal diazomethane was added to a 3ml glass reactivial containing either 10µg of authentic IAA or an unknown quantity of putative plant derived IAA dissolved in 100µl of methanol. The reactivial was then sealed and left at room temperature for five minutes.

After this period, the colour of the sample was observed. If the yellow colouration remained the diazomethane was still in excess and the reaction considered complete. The ether was evaporated from the vial under a stream of nitrogen in a fume hood. The sides of the vial were then washed with diethyl ether to reconcentrate the sample into the conical portion of the vial and the ether was then removed under a stream of nitrogen. The dried samples were stored at -20°C until required for

GC-MS analysis. This method of methylation was found to be 99% efficient for the derivatisation of standard IAA (Table 3.6).

GC-MS conditions

GC-MS analysis was performed on a Hewlett Packard 5890 combined GC-MS fitted with an on-line data processing unit (Hewlett Packard 7946, Hewlett Packard, Winnersh, Wokingham). Standard methyl IAA (1-5µl) was automatically injected, splitlessly, onto an OV1 Gas Chromatography column (25m x 0.2mm i.d., methyl silicone gum, 0.33µ film thickness, Hewlett Packard) with helium used as the carrier gas. Derivatised plant samples were manually injected onto the column in order to introduce as much of the sample as possible to aid in the detection of IAA. Temperature programming was from 55-280°C, the rate of temperature rise was 15°C/minute, starting 2 minutes after injection.

Samples were fragmented by 70 eV electron impact ionisation producing a positive ion spectrum. A full scan mass spectrum of standard methyl IAA produced by 70 eV electron impact ionisation on this instrument is illustrated in Figure 3.6. The mass spectrum trace demonstrates the presence of minor ions m/z 103, 77 and 51 resulting from further ionisation of the indole molecule (McDougall and Hillman 1978). The base ion (m/z 130) is the most abundant ion produced. The ratio of abundance of the

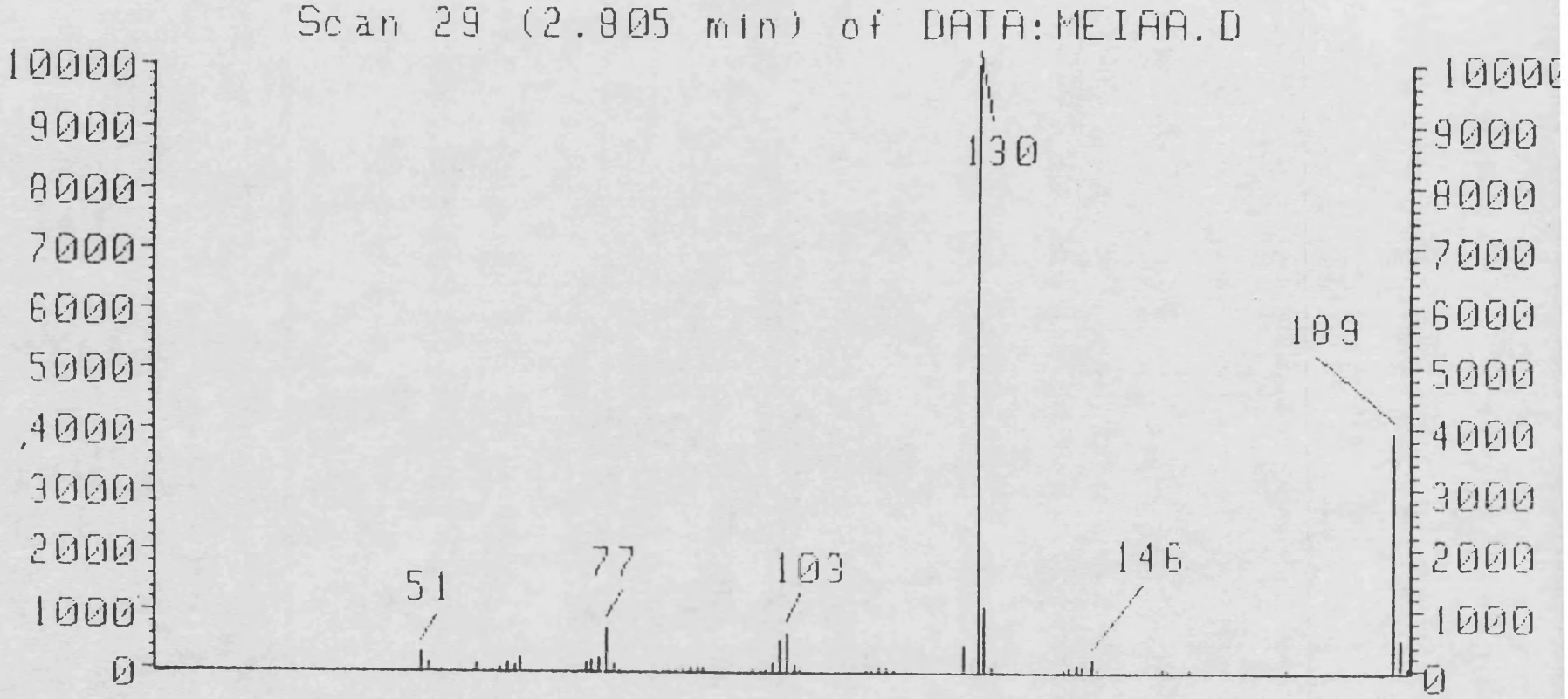


Figure 3.6 : Full scan mass spectrum of methyl IAA.

molecular ion (m/z 189) to the base ion (ion m/z 130) on mass fragmentation of authentic methyl IAA is used for the identification of IAA. The presence of the same ratio of ion abundance (m/z 189/130) in a plant sample to that of a standard methyl IAA trace at a coincident retention time may be taken as an identification of IAA in the sample.

Selective Ion Monitoring

The amount of methyl IAA required for mass spectral analysis, for both validation and quantification, is dependent upon the instrument in use. The sensitivity of GC-MS detection may be improved by employing selective ion monitoring (SIM), allowing the concentration of the electron capture detector on the ions of most interest in the sample. The use of the SIM mode ignores contaminant ions present in the extract but, by monitoring only specific ions in the sample, methyl IAA may be identified in an extract in amounts which would be undetectable using full scan mass spectrometry. The SIM mode on GC-MS was used throughout this work as the quantities of methyl IAA recovered from the plant extracts were not detectable using non-selective mass spectrometry on this instrument. On the SIM mode, the lowest amount of standard methyl IAA confidently detectable was 100pg (Figure 3.7).

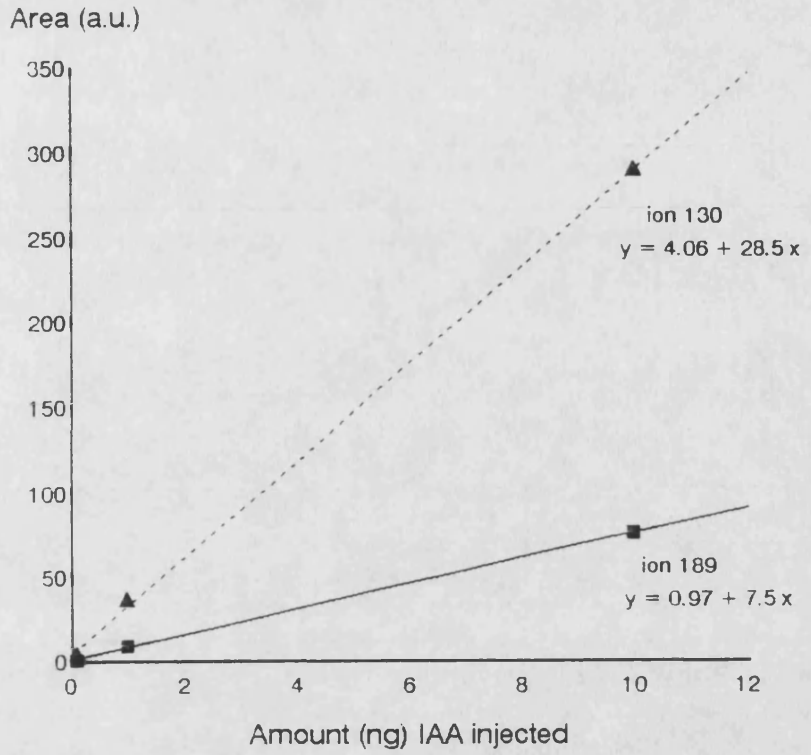
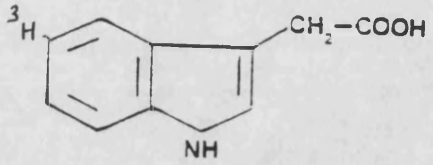


Figure 3.7 : Standard methyl IAA curve on GC-MS(SIM).

Quantification of IAA by HPLC-F

Internal standard



5-^{3}(H)IAA

5-^{3}(H)IAA was chosen as an internal standard for the estimation of IAA losses during the purification procedure. This compound has a high specific activity (925 GBq/mmol)

and the high counts associated with the low carrier weight of IAA was useful in this work as the weight of tissue taken for analysis was low. High specific activity in an internal standard allows the detection of trace amounts of the compound at the final stage of analysis without the initial addition of high quantities of IAA which could interfere considerably in the accurate determination of endogenous IAA levels.

5-^{3}(H)IAA has been reported to possess an enhanced possibility of breakdown in comparison to other forms of radiolabelled IAA such as (1-^{14}C)IAA and (2-^{14}C)IAA (Sandberg et al 1987). Despite this acknowledged lability, tritiated IAA is still in widespread use as an internal standard for the estimation of IAA recoveries from purification procedures (eg. Chen et al 1988, Pengelly et al 1986).

The radioactive purity of the internal standard was determined for each IAA analysis carried out in this work because of the labile nature of the labelled compound. The 5-^{3}(H)IAA was diluted in methanol and stored in 200µl aliquots at -20°C in sealed glass reactivials.

Determination of radioactive purity of internal standard (5-^{3}(H)IAA)

The radioactive purity of the internal standard was assessed by comparing the radioactive counts associated with the compound before and after HPLC purification. A known amount of 5-^{3}(H)IAA (1.42ng/20µl) was loaded onto a Spherisorb ODS1 (250 x 4.6mm) analytical HPLC column and

eluted isocratically under the conditions described above. The areas under the peaks produced on the chromatograms were integrated automatically by a CI-10B Milton Roy integrator. The entire peak was also collected in scintillation vials, to which Optiphase "Safe" scintillant (LKB) was added, for the determination of radioactivity in an LKB (Wallac) 1217 Rackbeta liquid scintillation counter. Quenching in the samples was measured internally by the channels ratio method and samples were appropriately corrected for quantification. The disintegrations per minute produced by the HPLC purified 5-^{3}(H)IAA were compared to those measured in an equivalent quantity of non-purified tritiated IAA. A percentage value of radioactive purity was obtained in ths way for the isotope for each IAA analysis carried out. The radioactive purity was then taken into account in the determination of endogenous IAA levels in the plant samples.

The calculated radioactive purities for the internal standard are presented in the Appendix (Appendix F) and the loss of isotopic purity of the 5-^{3}(H)IAA measured over a twelve week period is demonstrated in Figure 3.8. An HPLC profile of the tritiated IAA standard after six weeks in storage at -20°C is illustrated in Figure 3.9 and clearly shows the partially degraded nature of the compound.
The isotope arrived with a manufacturers (Amersham International plc.) reported purity of 97%. After dilution and storage of the isotope at -20°C, the maximum purity determined for the isotope was 81 %.

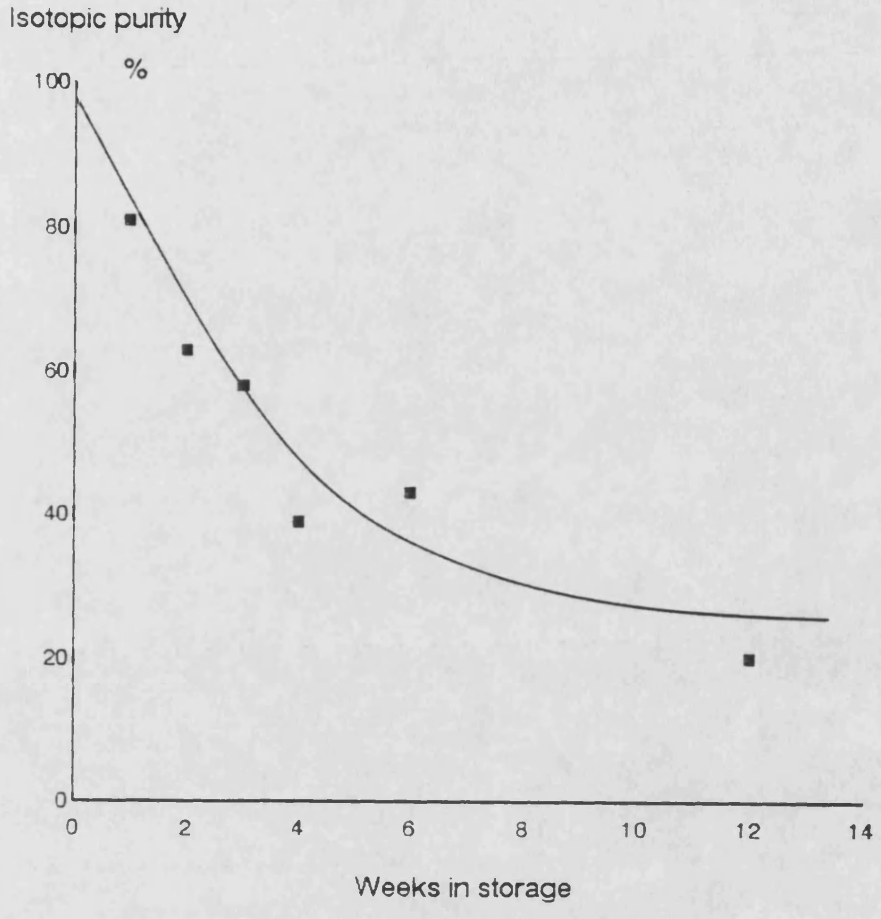


Figure 3.8 : Loss of isotopic purity of 5-^{3}(H)-IAA over a twelve week period in storage at -20°C.

Column : Spherisorb ODS1
(250 X 4.6mm)

Flow rate : 1ml/min

Detector : Fluoromonitor

Excitation 254nm

Emission 355nm

Eluant : 45% methanol in
1mM acetic acid

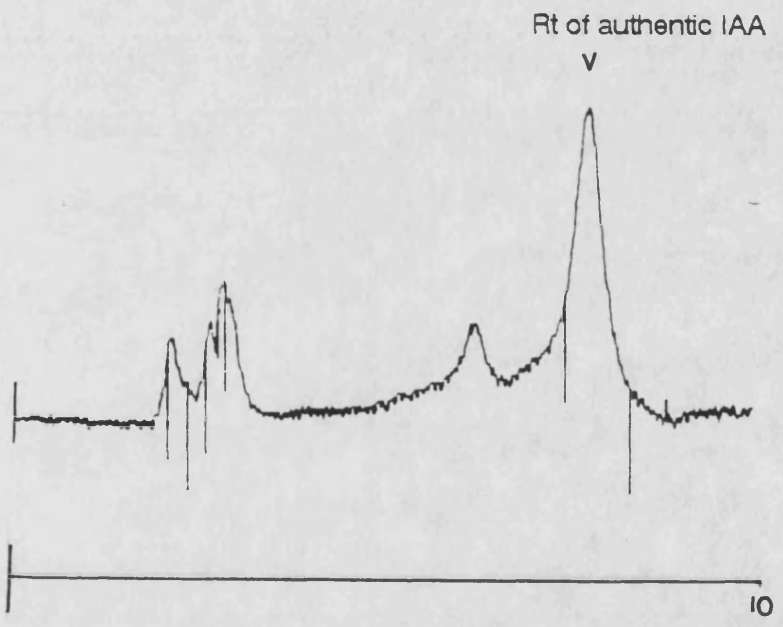


Figure 3.9 : HPLC chromatogram of 5-(H)-IAA after six weeks in storage.

Quantification of IAA by HPLC-F

Fluorescent peaks of retention times close to or co-incident with those of standard IAA were observed in all HPLC-F chromatograms obtained from the callus extracts (eg Figure 3.11). This putative IAA was quantified by collecting three replicate peaks from each of the three sample replicates of each plant extract for radioactivity determinations. These peaks were collected in scintillation vials by hand to exclude any contaminating radioactivity from outwith the IAA peak which could have considerably affected the determination of radioactivity and therefore specific activity. The build up of radioactivity on the column was avoided by frequent washing of the column with eluant or 100% methanol between sample injections.

Radioactivity was determined as described above. The areas under the collected peaks were automatically integrated by an LDC/Milton Roy integrator and then converted to nanogram equivalents by comparison of these areas with those of a known amount of standard IAA (a mean value obtained from at least 10 replicate injections). Endogenous IAA levels in the callus extracts were quantified using the isotope dilution method of Rittenberg and Foster (1940).

$$Y = (Ci/ Cf - 1) X$$

where;

Y = amount of endogenous IAA extracted from the tissue

Ci = the initial specific activity of the internal standard added to the sample.

Cf = the specific activity after dilution with endogenous IAA

X = the amount of internal standard added to the sample.

The value of X was corrected for the decomposition of the internal standard calculated from the radioactive purity determined at the time of analysis. The specific activity of the internal standard (Ci) used in the quantification was a mean value of eight separate determinations. This specific activity was calculated to be 2.68×10^5 dpms/ng, which is lower than that reported by the manufacturer at 3.13×10^5 dpms/ng.

Quantification of IAA by GC-MS

Internal Standard

Dideuterated IAA has been employed extensively as an internal standard for the quantitative analysis of plant derived IAA by GC-MS (Caruso et al 1978, Palni and Summons 1987, McDougall and Hillman 1978). Dideuterated IAA is not the most stable form of deuterated isotope available for IAA as deuterium exchange has been shown to occur during

partitioning and purification procedures prior to GC-MS analysis (Caruso and Zeiser 1983). Tetra and penta-deuterated forms of IAA are being increasingly used in place of d_2 IAA due to the more stable incorporation of deuterium atoms into the benzene ring of the IAA molecule (Magnus 1980, Pengelly et al 1981) than its more labile side chain (Magnus 1980). $^{13}_{6}C$ IAA is also available and has been employed for the quantification of IAA by GC-MS (Cohen et al 1986, Chen et al 1988). This isotope is extremely stable and has even been sucessfully employed for the analysis of IAA conjugates which requires alkaline hydrolysis (Cohen et al 1986).

Dideuterated IAA is, however, the simplest form of deuterated IAA to produce in the laboratory with the majority of the deuterium incorporated into the methylene group of the side chain (Magnus et al 1980).

The deuteration of IAA

IAA was deuterated by the method of Hoskins and Pollitt (1975). 100mg of IAA was placed into a 3ml glass reactivial (Pierce Chemical Co.). 1.2ml of 10% $NaOD_2$ was added to the vial which was then sealed and placed in an autoclave at 120°C for 3 hours. The vial was then removed and the IAA solution transferred to a glass tube and acidified by the addition of 0.75ml of 25% HCl. The vial was then rinsed with pH 3 distilled, deionised water and the washings transferred to the IAA solution.

IAA was extracted from this solution by partitioning

twice against 5ml volumes of diethyl ether. The ethereal phases were pooled and evaporated to dryness in a glass tube under a stream of nitrogen. The IAA was then taken up in small volume of methanol and the solution made upto a volume of 10ml.

The deuterated IAA solution (10mg/ml) was stored at -20°C prior to its use as an internal standard for the quantitative estimation of endogenous IAA levels in callus extracts by GC-MS. The deuterated IAA was also used as a standard for calibration of the the GC-MS for quantification purposes. Allen et al (1979) has reported that dideuterated IAA remains stable under similar storage conditions in ethanol at -15°C for periods of upto three months.

Determination of the relative abundance of isotopic forms in the deuterated IAA standard

The percentage of dideuterated IAA in the deuterated IAA standard could not be determined directly using GC-MS integrated areas for ion abundance as the instrument failed to integrate the areas of certain ions which were of significant abundance in the mass spectrum of the d IAA (Figure 3.10). These ions must, however, be taken into account in the determination of the percentage of d_2 IAA in the internal standard. The relative abundance of the various forms of IAA present in the internal standard was determined by measuring the heights of ion abundance

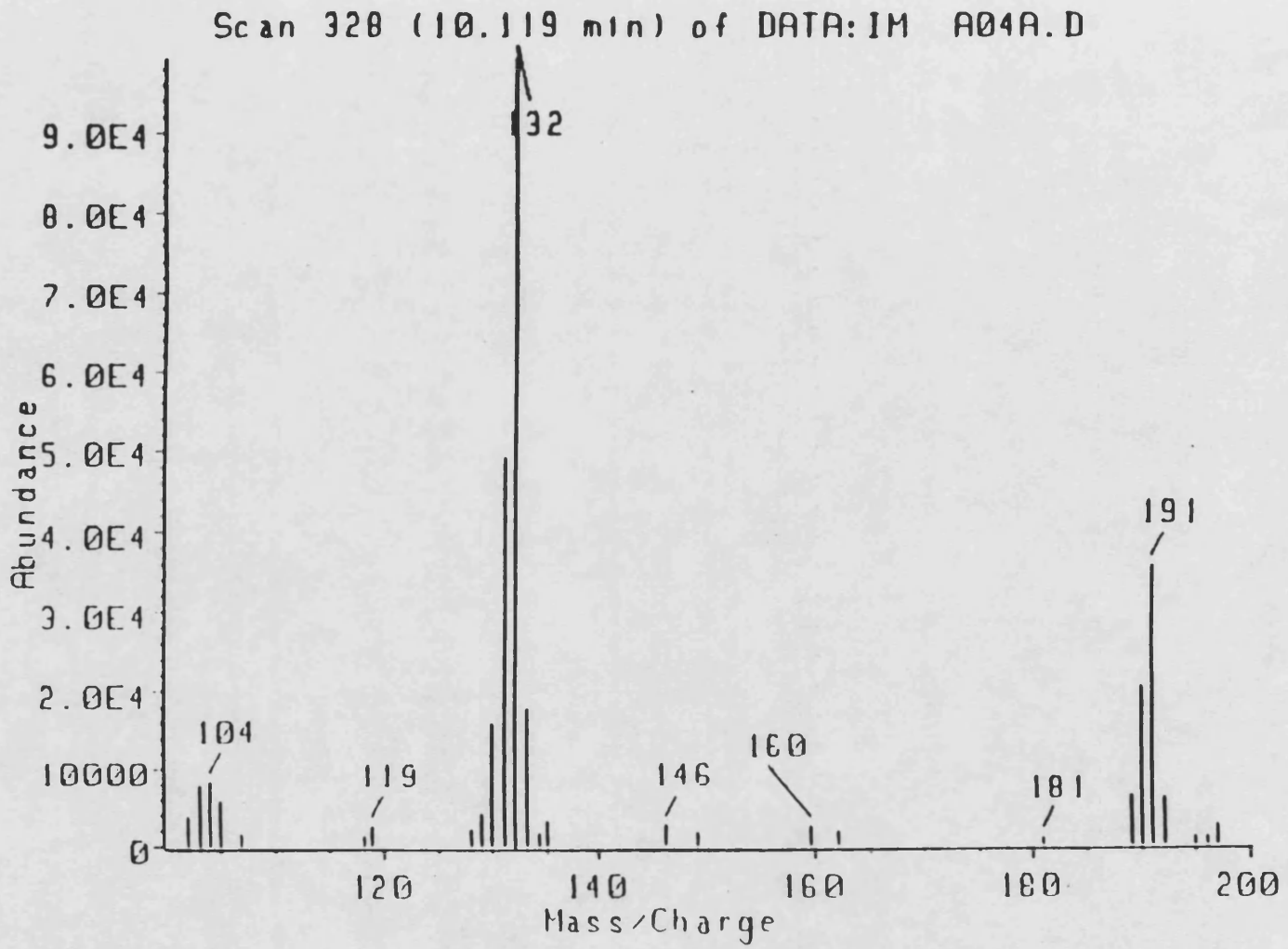


Figure 3.10 : Full scan mass spectrum of deuterated methyl IAA.

directly from the mass spectrum (Figure 3.10). GC-MS integrated areas were available for ions 130, 132, 189 and 191 and these values were used to check on the accuracy of the determinations made (Table 3.2). Table 3.1 shows the respective heights of the most abundant ions in the mass spectrum (ie. 189, 190, 191, 192, 130, 131, 132 and 133).

Base Ion	Height (mm)	Relative abundance %	Mol. Ion	Height (mm)	Relative abundance %	Mean %
130	17	8.8	189	7	9.5	9.2
131	52	26.8	190	22	29.7	28.3
132	106	54.6	191	38	51.4	53.0
133	19	9.8	192	7	9.5	9.7

Table 3.1 : The relative abundance of the major ions in the d_2 methyl IAA standard based on height measurement.

The deuterated standard IAA contained approximately 9%, 28%, 53% and 10% of non, mono, di and trideuterated IAA. These other forms of IAA were taken into account in the quantification of endogenous IAA. Areas for ions 130, 132, 189 and 191 were obtained in the GC-MS analysis and and are presented in Table 3.2.

The isotopic abundance of the various forms of IAA were determined on the basis of the base ions, rather than the molecular ions, as the height measurements for base

ions correlated well with the GC-MS integrated peak areas available. The relative percentage of non, mono, di and trideuterated IAA in the internal standard were therefore taken to be 9%, 27%, 55% and 10% respectively.

Base Ion	Abundance (Area a.u.)	Height (mm)	Mol. Ion	Abundance (Area a.u.)	Height (mm)
130	5.1	17	189	1.3	7
132	36.8	106	191	12.1	38
% 130/132	14.0	16.0	% 189/191	11.0	18.0

Table 3.2 : A check on the validity of the height measurements of base and molecular ions by comparison to GC-MS integrated areas.

The recovery of IAA from the deuteration process

The deuteration of IAA by the above process could result in the significant loss of IAA. The recovery of IAA from the deuteration process was determined as follows. An aliquot (50µg) of the deuterated IAA was methylated and analysed by GC-MS. The total abundance of all of the most significant base ions present in the mass spectrum of the d IAA (130, 131, 132 and 133 (Fig. 3.10) was compared to the abundance of ion m/z 130 obtained from mass spectrometry of a known quantity (50ng/µl) of standard methyl non deuterated IAA. A high concentration (100ng/µl)

of IAA was deuterated for comparison with the standard IAA in anticipation of high loss. 0.1ml of the deuterated mixture (corresponding to 10mg of IAA) was removed from the standard solution and diluted to a volume of 1ml of methanol. 50µl of this solution (50ng, before allowing for loss) was removed and derivatised by diazomethane in a 3ml reactivial. The derivatised d_2 IAA was then taken up in 0.5ml of methanol to give a final concentration of 100 µg/ml, before loss. 2µl of this solution was injected into the GC-MS and analysed by the conditions described above. A full scan mass spectrum of the d_2 IAA was obtained (Fig. 3.10) but integrated areas were only obtained for four ions (m/z 130, 132, 189 and 190). These areas are presented in Table 3.2.

The recovery of IAA was then determined as follows;-

$$\text{Recovery of IAA} = \frac{\text{Area ion abundance } d_2\text{IAA-Me /100ng}}{\text{Area ion abundance IAA-Me / 100ng}}$$

$$\frac{3.37 \times 10}{5.00 \times 10} = 67\%$$

Standard	Ion Area	Abundance (a.u./100ng)
$d_2$methyl IAA	132	1.84
$d_2$methyl IAA	132 x 1.82	3.34
methyl IAA	130	5.0

Table 3.3 : GC-MS integrated areas for base ions in authentic standard methyl IAA and the internal standard $d_2$methyl IAA.

The total IAA in the internal standard (Table 3.3) was taken as the sum of the most abundant forms of IAA (ie. base ions 130, 131, 132 and 133). This was achieved by multiplying the integrated area of ion 132 in the internal standard by 1.82 (100/ 55, only 55% of the d_2IAA was dideuterated).

3.3 Results of HPLC-F estimations of endogenous IAA

HPLC-F profiles of the callus extracts (eg. Fig 3.11) demonstrated the presence of fluorescent peaks which were of retention times close to or co-incident with those of standard IAA. These peaks were typically well resolved and without any obvious signs of contamination in both the habituated and non-habituated callus extracts.

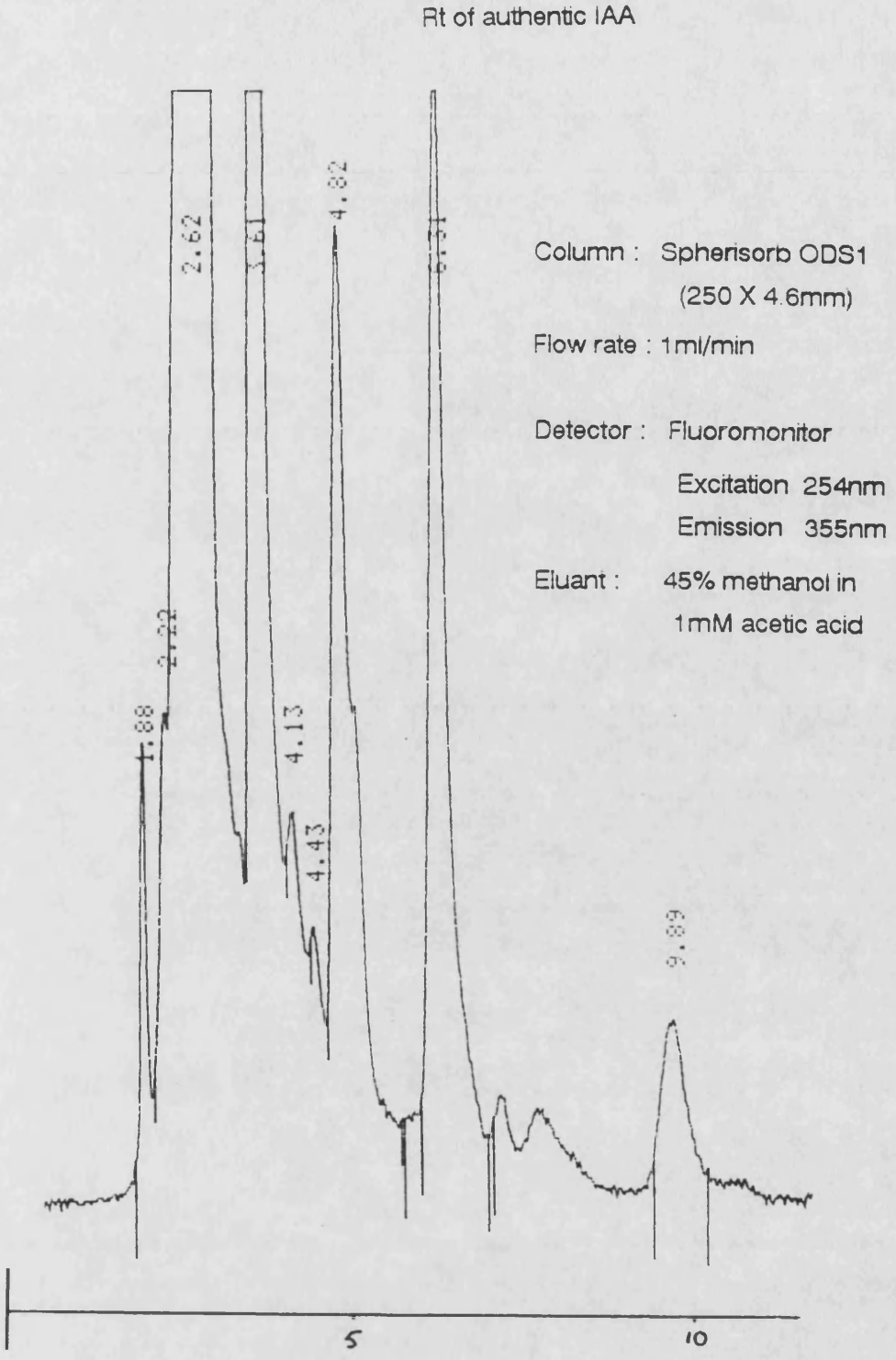


Figure 3.11 : HPLC chromatogram of 14 day old habituated extract purified by the above technique.

All of the putative IAA peaks recovered were found to contain radioactivity, indicating the recovery of IAA from the purification procedure. The recovery of radioactivity was, however, generally low and variable with a maximum recovery of 54% (Table 3.4). This scale of loss during purification is acceptable when the internal standard has been introduced at the extraction stage of the purification and remained detectable at the final stage of quantification. 5-^{3}(H)IAA was detected in all the IAA peaks recovered.

Days in culture	Recovery range (%)	
	Habituated	Non-habituated
7	0.2 - 0.7	15 - 17
14	4 - 12	21 - 33
21	8 - 11	22 - 54

Table 3.4 : The percentage recoveries of 5-^{3}(H)IAA from the purification of endogenous IAA from callus tissues. Figures represent the range of recoveries from three replicates.

These present results would not recommend the use of 5-^{3}(H)IAA as an internal standard for the estimation of IAA levels in plant tissues because of the highly labile nature of the ^{3}(H)-labelled compound, even when stored at -20°C

(Figures 3.8 and 3.9). The partially degraded nature of the isotope introduced problems in quantification and putative IAA peaks were collected manually to ensure that only radioactivity from the actual peak was measured. The potential for inaccuracy involved in the use of 5-^{3}(H)IAA as an internal standard was found to outweigh the advantage of high specific activity offered by this isotope.

The levels of endogenous IAA quantified by HPLC-F were observed to vary dramatically within the 21 day culture period in both the habituated and the non-habituated tissues (Table 3.5). There was also a noteable difference in the extent of fluctuation in IAA levels in the two tissues. In the habituated culture levels were seen to vary by approximately 10 fold, whilst the fluctuation measured in the non-habituated tissues was in excess of 100 fold. In the absence of IAA estimates at days directly on either side of the peak in IAA levels in the tissues, the actual maximum level of endogenous IAA in the tissues was not determined. The results do, however, suggest that the levels of IAA in the two tissues were of the same order of magnitude, despite the more dramatic fluctuation in levels measured in the non-habituated tissues during the growth cycle.

The highest level of IAA measured in the habituated culture (555ng/g) was observed 7 days into the culture period while the highest level in the non-habituated tissue (532ng/g) was seen after 14 days. This difference may have been related to the slower growth rate of the non-

habituated culture (doubling time of 5 days compared to 4 days in the habituated tissue), although no direct correlation between IAA levels and stage of growth was determined.

Days in culture	IAA levels (ng/g fwt ± SEM)	
	Habituated	Non-habituated
7	556 ± 145	5 ± 0.03
14	67 ± 16	531 ± 66
21	36 ± 13	10 ± 2

Table 3.5 : HPLC-F estimates of endogenous IAA levels in habituated and non-habituated Lactuca callus tissues.

3.4 Derivatisation of "IAA" peak recovered from HPLC-F of plant extracts

An attempt to determine the homogeneity of the putative IAA peaks recovered from the purification procedure was made using derivatisation. Law and Hamilton (1982) have employed this method of methylating purified extracts to confirm the identities of putative IAA and IAAsp isolated from Pisum sativum seedling tissue.

The putative IAA recovered from the purification of Lactuca callus tissues, after a preparative HPLC separation, was methylated by diazomethane and then loaded onto a preparative HPLC column (Spherisorb ODS1 25 x 10mm i.d.). The column was eluted under the HPLC conditions described above. No internal standard was employed and only the relative percentage of the putative IAA which co-chromatographed with authentic methyl IAA was estimated for each tissue. These estimates were based on the assumption that IAA and methyl IAA fluoresced with equivalent intensity, although this was not confirmed experimentally.

The retention times of authentic IAA and methyl IAA were first obtained by injecting methylated authentic IAA standard and these were found to be approximately 8 and 17 minutes respectively. Areas under the fluorescent peaks were integrated by a LDC/Milton Roy integrator. The methylation of standard IAA was found to be 99% efficient based on the percentage of the samples which methylated using peak areas (Table 3.6).

Column : Spherisorb ODS1
(250 x 10mm)
Flow rate : 4ml/min
Detector : Fluoromonitor
Excitation 254nm
Emission 355nm
Eluant : 45% methanol in
1mM acetic acid

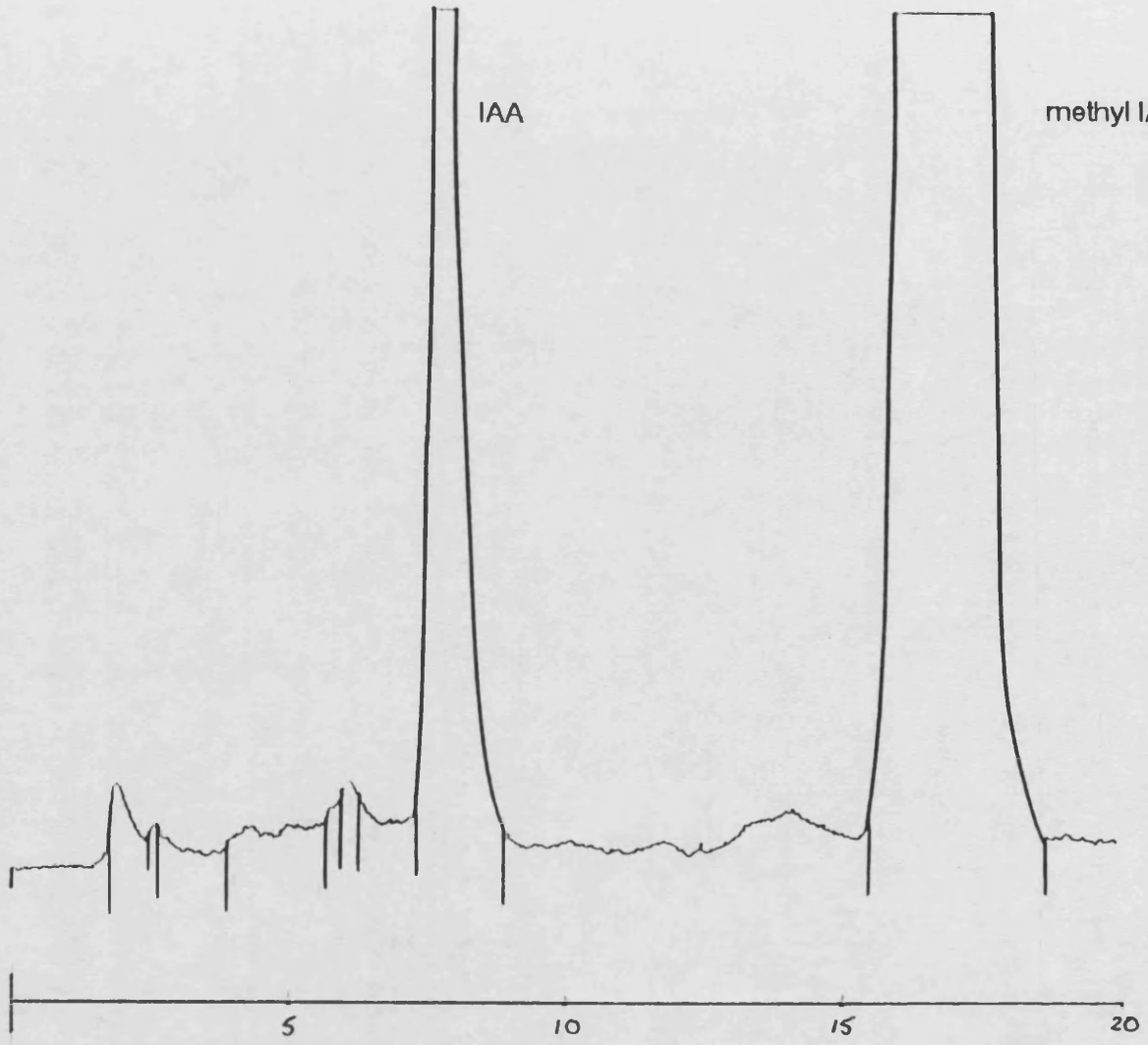
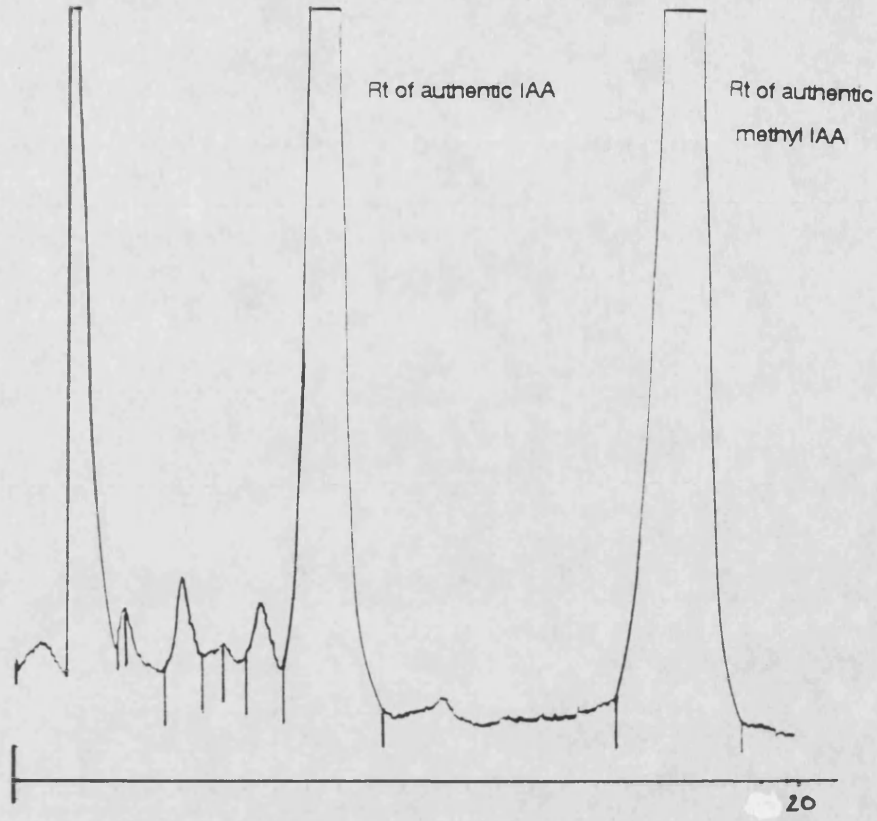
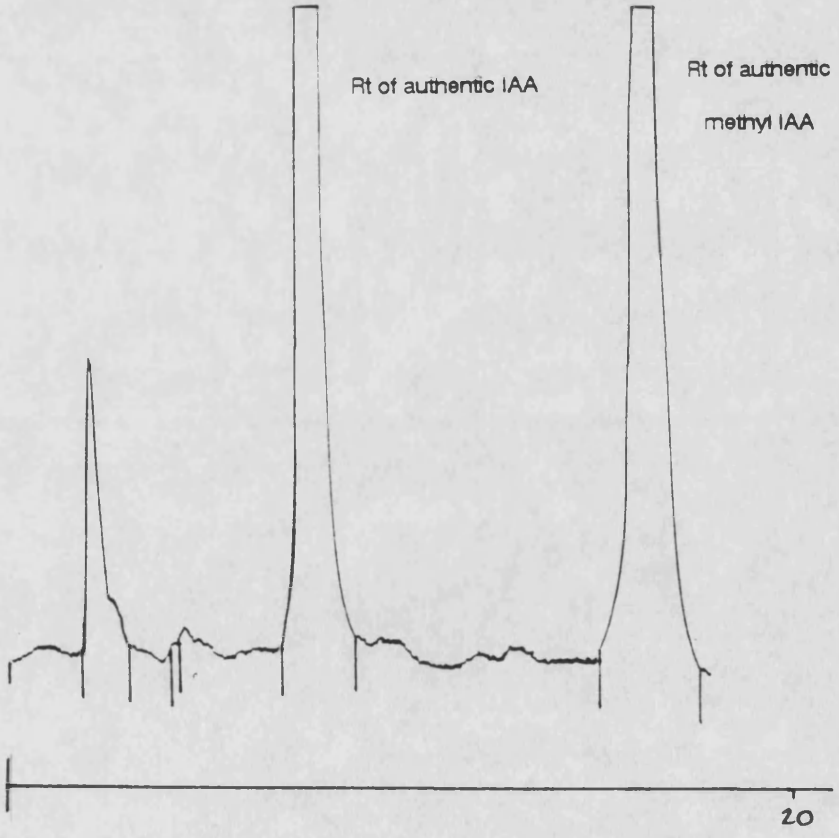


Figure 3.12 : HPLC chromatogram of authentic IAA derivatised by diazomethane.

Figures 3.13 and 3.14 : HPLC chromatograms of plant derived "IAA" from habituated (Fig. 3.13) and non habituated (Fig. 3.14) tissues. Chromatographic conditions as for Figure 3.12.


Rt of authentic IAA
Rt of authentic
methyl IAA
20


Rt of authentic IAA
Rt of authentic
methyl IAA
20

In the chromatograms of derivatised IAA purified from plant extracts, only those fluorescent peaks of retention times equal to or greater than that of authentic IAA (ie > 8 minutes) were considered in the estimation of purity as methylation could only increase the retention time of compounds on this column by decreasing their polarity.

Sample	Area (a.u.) Rt authentic methyl IAA	Area (a.u.) total fluorescence	% methylation
Habituated	7.24	9.01	80
	1.16	1.70	68
	3.04	5.06	60
	0.26	0.41	64
	0.12	0.23	52
		$\bar{x}$ 5 =	65%
Non-habituated	2.05	4.02	51
	1.73	4.21	41
		$\bar{x}$ 5 =	46%

Table 3.6 : Determination of the percentage fluorescence in the "IAA" peak which, after derivatisation co-eluted with authentic methyl IAA.

The fluorescent peaks present in the habituated and non-habituated methylated plant extracts are shown in Figures 3.13 and 3.14. Peaks prior to 8 minutes were not

found to be of significant fluorescence and no further significant peaks were observed after the retention time of authentic methyl IAA.

In the habituated extract, 65% of the fluorescence detected at 8 minutes and above was in the position of authentic methyl IAA (Table 3.6). This figure was only 46% in the non-habituated tissue. With the knowledge that the methylation of authentic IAA was 99% efficient, the prominant peaks in the plant extracts of retention times co-incident to standard IAA were unlikely to be IAA or indeed other carboxy acids which methylate readily (Cohen 1984). These peaks appear to indicate considerable contamination in the putative IAA peaks recovered from the plant tissue and quantified by HPLC-F.

These results clearly show that the IAA levels estimated by the single HPLC-F step were overestimates and, of perhaps even greater importance, was the suggestion that the degree of contamination present in the IAA recovered from the two tissue types was different.

Estimations of IAA levels by HPLC-F were recalculated based on the above results. This was achieved by reducing the HPLC-F integrated areas of the habituated tissues by 35% and the non-habituated areas by 55%. The IAA estimates corrected for overestimation (Table 3.7) now show maximum levels of 362 ng/g fwt in the habituated tissue and 247 ng/g fwt in the non-habituated tissue. Despite wide variation between replicate estimates these levels were significantly different. The levels of IAA measured in the

non-habituated tissue at day 7 and 21 were now extremely close to the detection limit of HPLC-F. This would undoubtedly have caused problems in quantification.

It must also be considered that the fluorescent peaks which co-chromatographed with methyl IAA may not be totally free of contamination after derivatisation and this second HPLC step. For this reason, a GC-MS confirmation of the presence of IAA in the extracts was necessary.

Sample/ Days in culture	IAA levels (ng/g fwt +/- SEM) Before correction	After correction
Habituated		
7	556 +/- 145	355 +/- 94
14	67 +/- 16	43 +/- 10
21	36 +/- 13	23 +/- 8
Non-Habituated		
7	5.1 +/- 0.03	1.3 +/- 0.02
14	531 +/- 66	230 +/- 20
21	10 +/- 1.7	6.4 +/- 1

Table 3.7 : Corrected estimates of endogenous IAA levels in callus tissue extracts. The correction factor was applied to each of the three areas integrated from each replicate.

3.5 Confirmation of the presence of IAA in habituated Lactuca callus extracts

GC-MS was employed to confirm the presence of IAA in the callus extracts recovered from the purification procedure. 12g fresh weight of ~~14~~ 7 day old habituated callus tissue was extracted and purified by the technique described above. No internal standard was employed as the purpose of the experiment was identification and not quantification.

The putative IAA recovered from a preparative HPLC-F separation was derivatised by diazomethane and then taken up in a minimal volume of methanol (20µl) to maximise the possibility of detecting any IAA present. A preliminary GC-MS run of the plant sample indicated that the level of IAA recovered was too low for detection by full scan mass spectral analysis. The SIM mode of GC-MS was therefore used throughout the analysis of both standard methyl IAA and the plant extract.

The dominant ions of methyl IAA (m/z 189 and 130) were monitored on the SIM program. A prominant peak on the GC trace was observed in the plant extract at a coincident retention time to that of standard methyl IAA (11.34 minutes, Figures 3.16 and 3.17). The ions m/z 189 and 130 were both of significant abundance in this peak. The results showed the presence of an identical ratio of relative ion abundance for ions m/z 189 and 130 in the plant extract and in standard methyl IAA, providing strong evidence for the presence of IAA in the habituated tissue.

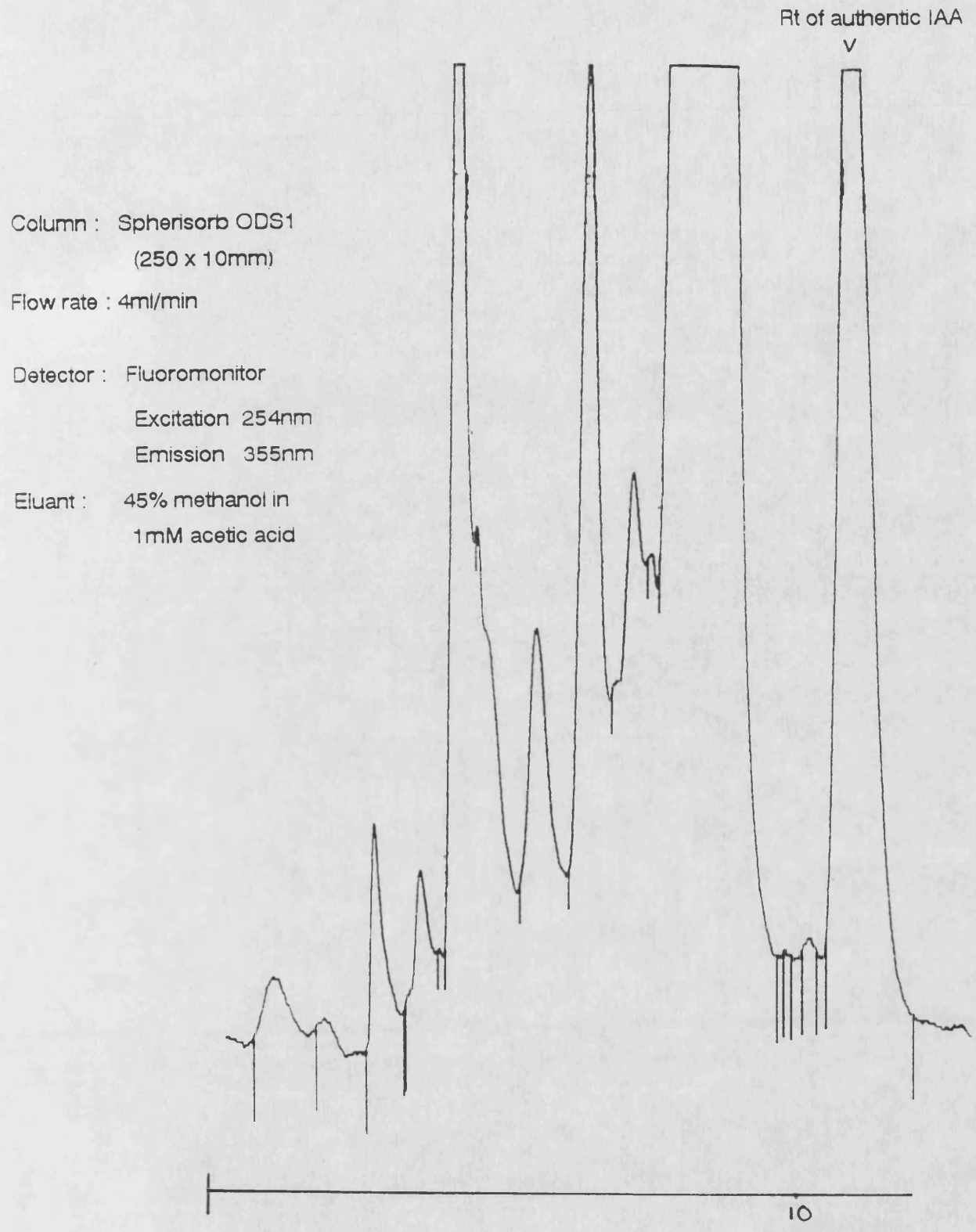


Figure 3.15 : HPLC chromatogram of the habituated extract purified for GC-MS(SIM) analysis

Figures 3.16 and 3.17 : GC-MS(SIM) traces of ions 189 and 130 in authentic methyl IAA (Fig. 3.16) and in a habituated sample (3.17).

Ratio of ion abundance 189/130 :

Authentic IAA (Fig.3.16) : 31%

Habituated sample (Fig. 3.17) : 31%

Chromatographic conditions :

Column : OV1 , methyl silicone gum, (25m x 0.2mm i.d.)

Temp. programming : 55 -280 c at rate rise of 15 c/min.

Carrier gas : Helium

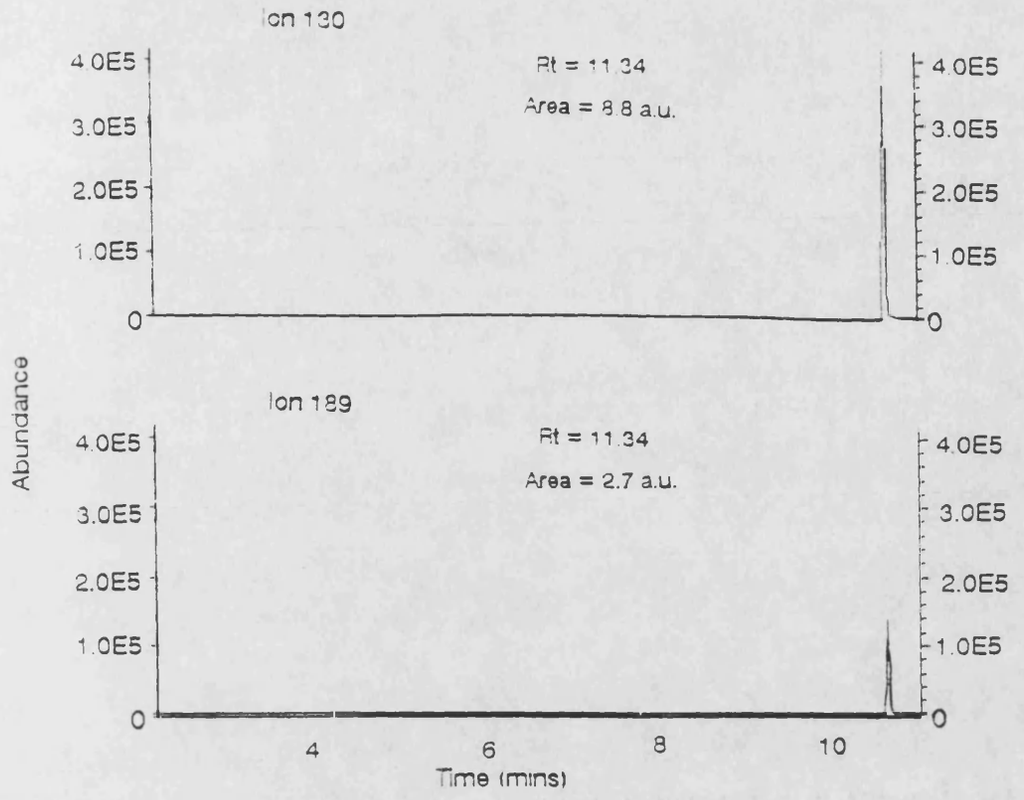


Figure 3.16

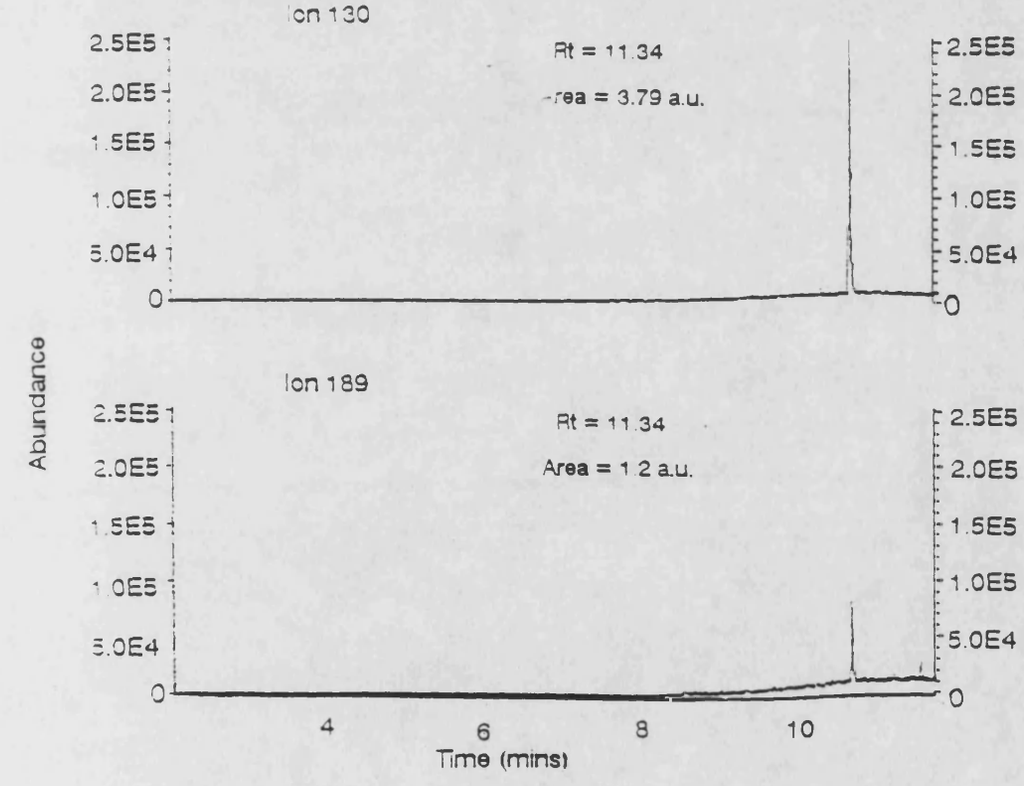


Figure 3.17

Figures 3.18 and 3.19 : GC-MS(SIM) traces of ions 189 and 130 in a habituated sample (3.18) and in a non habituated sample (3.19).

Ratio of ion abundance 189/130 :

Habituated sample (3.18) : 32%

Non habituated sample (3.19) : 33.5%

Chromatographic conditions :

Column : OV1 , methyl silicone gum, (25m x 0.2mm i.d.)

Temp. programming : 55 -280 c at rate rise of 15 c/min.

Carrier gas : Helium

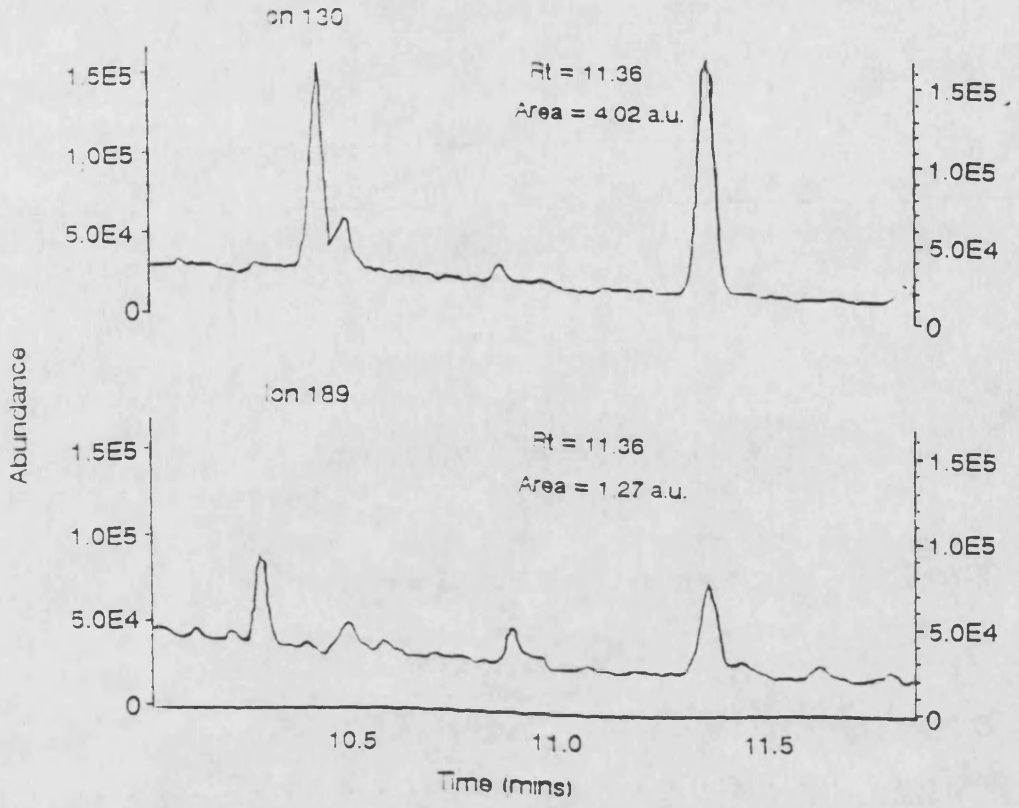


Figure 3.18

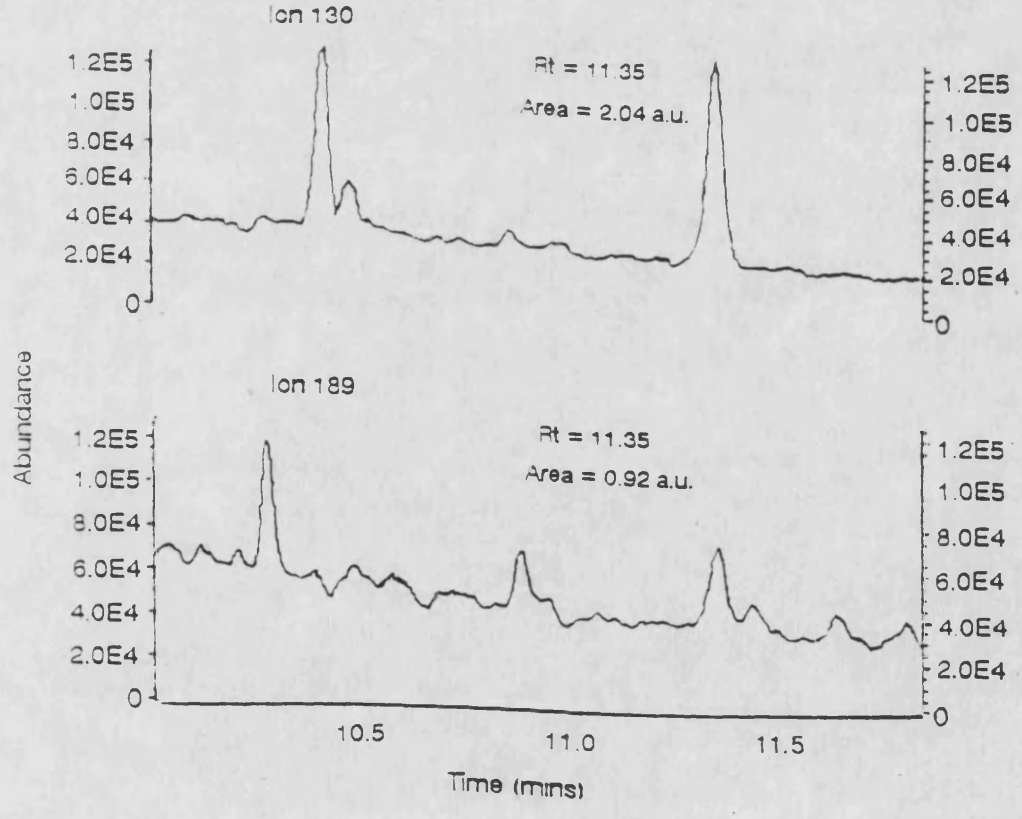


Figure 3.19

The GC-MS (SIM) traces of ions m/z 189 and 130 in the plant extract were low in background contamination (Figure 3.17).

Figures 3.18 and 3.19 demonstrate the presence of significant quantities of ions m/z 130 and 189 in both habituated and non-habituated extracts at the retention time of authentic methyl IAA on a second GC-MS (SIM) analysis of plant tissue samples. The ratio of ions m/z 189 to 130 in these samples were found to be 32% in the habituated extract and 33.5% in the non-habituated extract, within the ratios expected for authentic methyl IAA on several preliminary GC-MS runs (Appendix G).

They were not, however, identical to the ratio obtained for standard methyl IAA on the day of analysis. The GC traces show significant contamination in all of the ions monitored on SIM mode in this second analysis. The results from this second GC-MS(SIM) analysis may, therefore, only be taken as preliminary evidence of the existence of IAA in the plant extracts. The above evidence indicating that IAA was present in the habituated extract (Fig. 3.17) supported the possibility that IAA was also present in these plant extracts in this second analytical run but was masked by contamination.

3.6 Quantification of IAA by GC-MS(SIM)

Dideuterated IAA was used as an internal standard for the estimation of endogenous IAA levels in callus extracts of habituated and non-habituated Lactuca tissues. Putative IAA was recovered from a preparative HPLC-F separation following purification by the above technique. This IAA was methylated and taken up in 20µl of methanol. A 3µl aliquot of each plant extract (habituated and non-habituated) was then manually injected onto the GC column.

Standard methyl IAA gave a retention time of 11.4 minutes under the conditions described above. Prominant GC peaks of a coincident retention time were observed in the deuterated IAA standard (Figure 3.20) and in both the habituated and non-habituated plant extracts (Figures 3.21 and 3.22). Considerable background contamination of the selected ions was present in all of the traces obtained.

The amount of IAA recovered from the plant extracts was again too low to obtain a full scan mass spectrum of the putative methyl IAA peaks on the GC traces of the plant extracts and SIM was used for this analysis with ions m/z 130, 131, 132, 189, 190 and 191 monitored as the ions of interest in the samples. GC-MS(SIM) integrated areas for the abundance of these ions in standard d_2 methyl IAA and the plant samples are presented in Table 3.8.

The results show that the ratios of molecular to base ion abundance, typically employed for the identification of methyl IAA were extremely variable and generally high in both standard d_2 methyl IAA and in the plant extracts.

Figures 3.20, 3.21 and 3.22 : GC-MS(SIM) traces of ions 189, 130, 191 and 132 in authentic IAA (Fig. 3.20), a habituated sample (Fig.3.21) and in a non habituated sample (3.22).

Chromatographic conditions :

Column : OV1 , methyl silicone gum, (25m x 0.2mm i.d.)

Temp. programming : 55 -280 c at rate rise of 15 c/min.

Carrier gas : Helium

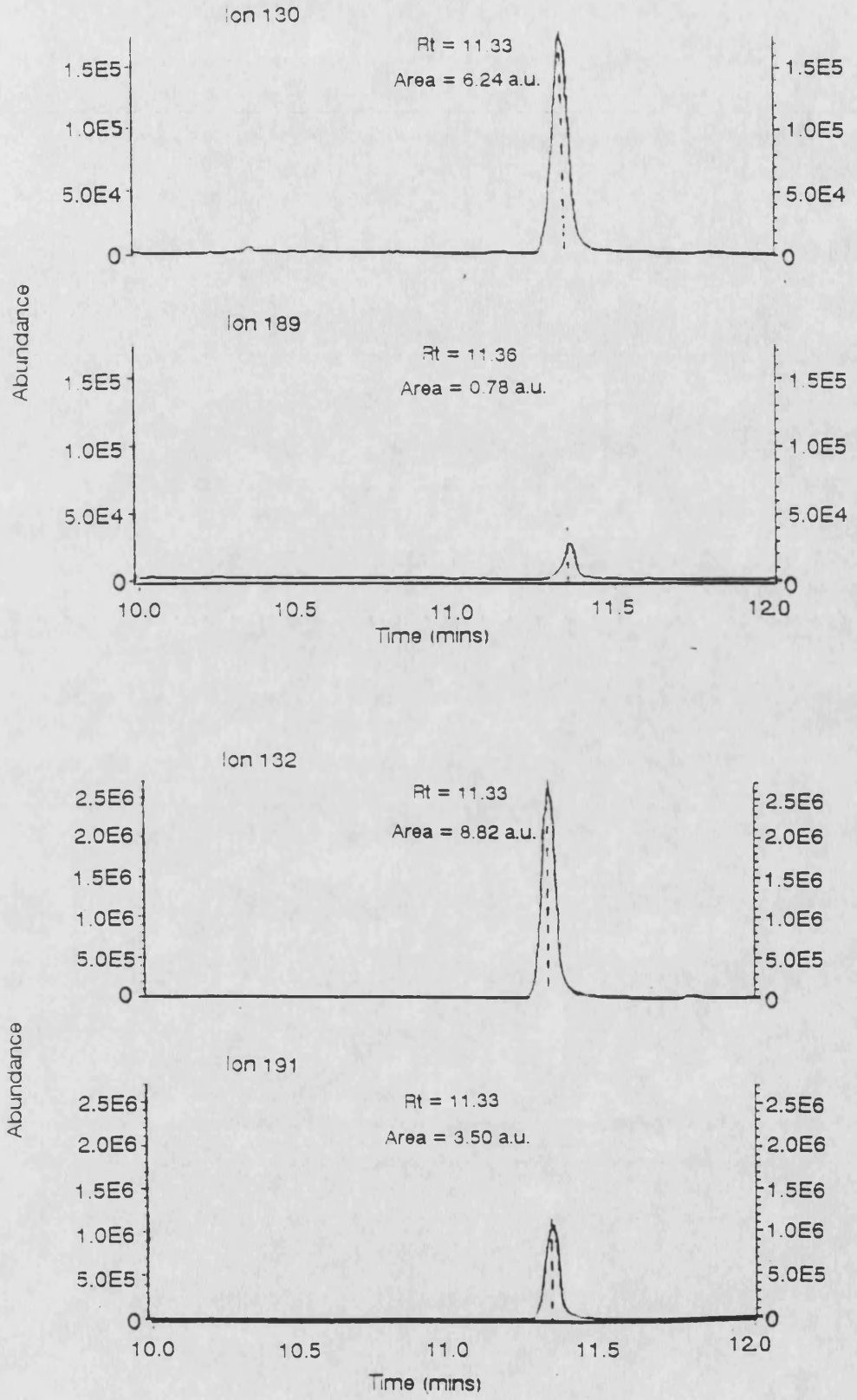

Ion 130
Rt = 11.33
Area = 6.24 a.u.
1.5E5
1.0E5
5.0E4
0
Ion 189
Rt = 11.36
Area = 0.78 a.u.
Abundance
10.0
10.5
11.0
11.5
12.0
Time (mins)
Ion 132
Rt = 11.33
Area = 8.82 a.u.
2.5E6
2.0E6
1.5E6
1.0E6
5.0E5
Ion 191
Rt = 11.33
Area = 3.50 a.u.

Figure 3.20

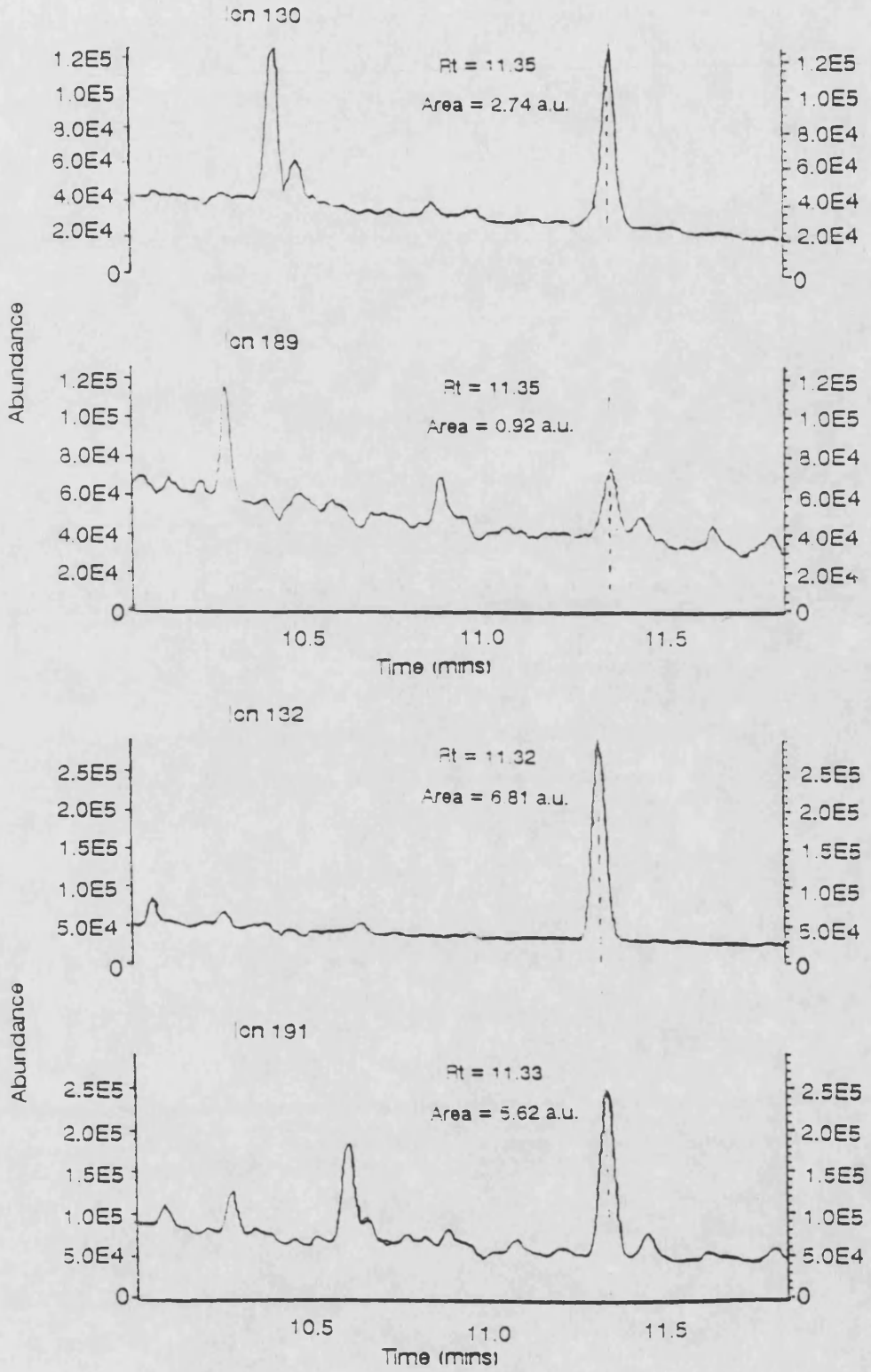

Ion 130
Rt = 11.35
Area = 2.74 a.u.
1.2E5
1.0E5
8.0E4
6.0E4
4.0E4
2.0E4
0
Ion 189
Rt = 11.35
Area = 0.92 a.u.
Abundance
10.5
11.0
11.5
Time (mins)
Ion 132
Rt = 11.32
Area = 6.81 a.u.
2.5E5
2.0E5
1.5E5
1.0E5
5.0E4
Ion 191
Rt = 11.33
Area = 5.62 a.u.

Figure 3.21

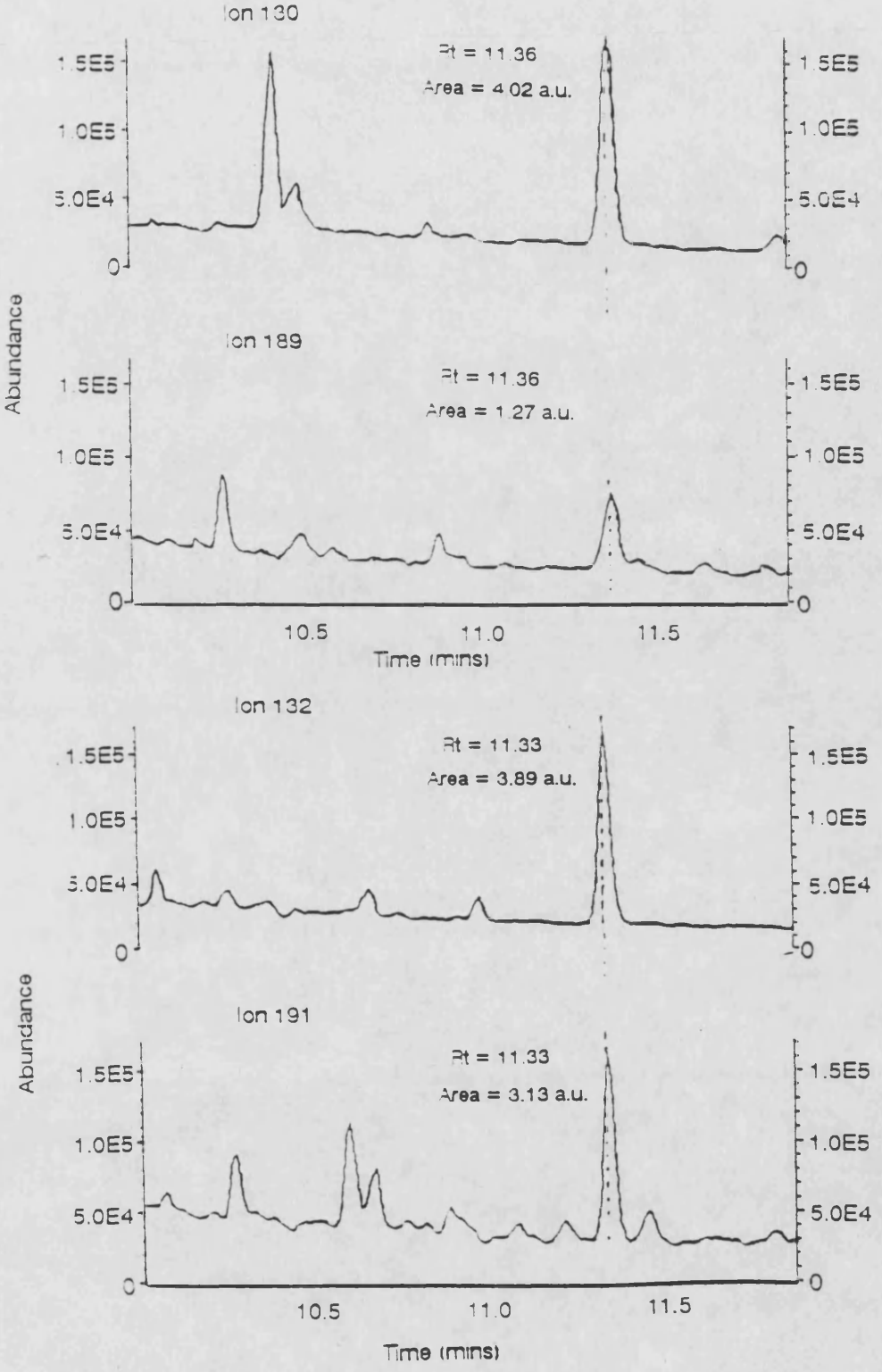

Ion 130
Rt = 11.36
Area = 4.02 a.u.
1.5E5
1.0E5
5.0E4
0
Ion 189
Rt = 11.36
Area = 1.27 a.u.
Abundance
10.5
11.0
11.5
Time (mins)
Ion 132
Rt = 11.33
Area = 3.89 a.u.
Ion 191
Rt = 11.33
Area = 3.13 a.u.
Abundance
10.5
11.0
11.5
Time (mins)

Figure 3.22

Preliminary GC-MS runs of methyl IAA provided molecular to base ion ratios within the range of 26-36% (Appendix G). A preliminary GC-MS run of the internal standard used in this analysis also gave a molecular to base ion abundance within this range (ie. 26% for m/z 189/130 and 33% for m/z 191/132, Appendix G). These values were significantly different from those obtained for authentic IAA in this analysis.

	Abundance (Area a.u.)		
	Standard	Habituated	Non-habituated
Ion	d_2 methyl IAA	sample	sample
189	7.8	12.7	9.2
130	62.4	40.2	27.4
Ratio $\frac{189}{130}$	12.4	31.7	33.5
191	350	31.3	56.2
132	882	38.9	68.1
Ratio $\frac{191}{132}$	39.7	80.6	82.6

Table 3.8 : GC-MS integrated areas for the abundance ofions m/z 130, 132, 189 and 191 monitored in standard d_2 methyl IAA and Lactuca callus extracts on (SIM) mode.

The unusual ratios of ion abundance obtained were undoubtedly the result of contamination of the GC column or of the extracts (including the methyl IAA standards). The pattern of variation in the ratios between non, mono and dideuterated forms of IAA within the d_2 methyl IAA standard were reflected in the ratios obtained from the plant samples. Certain molecular to base ion ratios were more unusual than others, suggesting the contamination of specific ions in the traces.

Ion abundance ratios in the plant samples were always higher than the respective ratio in the d_2 methyl IAA standard and there was a general trend of increasing molecular to base ion abundance ratios with the extent of deuteration. The higher ratio in the dideuterated form might suggest a less efficient fragmentation of the molecular ion or may be an artefactual difference exaggerated by a lack of replication possible in this work. If inefficient fragmentation of the d methyl IAA was a problem, a more accurate estimation of IAA levels may be obtained based on the joint abundance of molecular and base ions.

The presence of unusual molecular to base ion abundance ratios throughout this analysis was undoubtedly the result of contamination. The nature of the contamination problem was not, however, established. From the apparent reflection of the variation in molecular to base ion ratios of d_2 methyl IAA standards in the plant extracts, an estimate of IAA levels in the plant tissues

could be obtained from the data. The results may, however, only be considered as a preliminary GC-MS quantification of IAA levels in the plant tissues and their interpretation must be limited due to the presence of contamination and a lack of replication.

From the areas of ion abundance obtained in the d_2 methyl IAA standard and the plant samples, the initial percentage of dideuterated IAA (Ci) in the internal standard and the percentage of dideuterated IAA recovered (Cf) in the plant samples were determined based upon either molecular ion areas (ions m/z 189 and 191), base ion areas (m/z 130 and 132) or the joint abundance of both (ie. m/z 130+189 and 132+191)(Table 3.9). Endogenous IAA levels in the plant samples were then estimated by the isotope dilution method (Rittenberg and Foster 1940).

$$0.89Y = (Ci/Cf-1)\ X$$

$$0.89\ Y = \left(\frac{\frac{132}{130 + 132}}{\frac{132}{130 + 130 + 132}} - 1 \right) X$$

where;

Y = amount of endogenous IAA in the tissue

Ci= the initial percentage of d_2IAA in the internal standard

Cf= the percentage of d_2IAA found in the sample

X = the amount of d_2IAA added

63.4% of the internal standard was calculated to contain non and dideuterated IAA (see Table 3.1). The value of X was therefor calculated as 3015ng X 0.63 = 1900ng. The value of 0.89 is the percentage of the endogenous IAA which contains all of the different isotopic forms of IAA.

	% Dideuterated IAA		
Sample	Base Ion	Molecular Ion	Joint
d_2 methyl IAA (Ci)	93	98	95
Habituated sample (Cf)	49	71	57
Non-habituated sample (Cf)	71	86	77
IAA levels (ng/g fwt) Habituated	96	41	71
Non-habituated	33	15	25

Table 3.9 : The percentage of dideuterated IAA recovered in standard and plant extracts based on GC-MS(SIM) integrated areas and the levels of endogenous IAA estimated based on these values.

A significant discrepancy was observed in the levels of IAA estimated using the abundance of molecular ion areas and those estimated using base ion areas. This variation reflected the problematic ratios of molecular to base ion abundance obtained. Estimations of IAA based on the abundance of the base ions (m/z 189 and 191) should, ideally, provide a check on the accuracy of the estimations based on the molecular ion areas (ie m/z 130 and 132) but this was not possible due to problems of contamination.

An attempt was made to identify those ions responsible for the observed contamination. As mentioned above, the percentage of d_2IAA in the internal standard should remain unchanged in methanolic solution at -20°C. (Allen et al 1979) have reported the stability of dideuterated IAA stored in ethanol at -15°C for a period of upto 3 months. The ratios of non/dideuterated IAA were compared in two separate GC-MS analyses of the same internal standard. A preliminary GC-MS analysis of the d IAA internal standard provided an apparently normal ratio of molecular/base ion abundance and this was taken to be an accurate measure of ion abundances in the internal standard.

The relative ratio of abundance of ions m/z 130:132 in this accurate analysis was 14%. On the second analysis when unusual molecular to base ion areas were obtained this ratio had fallen to 7% by either the contamination in ion m/z 132 or the underestimation of ion 130. Similarly, the relative areas of the ions (m/z 189:191) in the first analysis was 11% and this ratio fell to only 2% in the

second analysis by the overestimation of ion 191 or underestimation of ion m/z 189. The more pronounced difference between these non/dideuterated forms of IAA in the molecular ion areas suggested greater problems in the accurate quantification of these ions. These ions are present in the GC-MS analysis in much lower abundance than the base ions and this inevitably results in less accurate measurements of ion abundance.

	IAA levels (ng/g fwt ± SEM)	
Sample	GC-MS(SIM) n = 1	HPLC-F (corrected) n = 3
Habituated (7 day old)	96	355 ± 94
Non-habituated (14 day old)	33	230 ± 20

Table 3.10 : Comparison of corrected HPLC-F and GC-MS(SIM) estimations of endogenous IAA levels in habituated and non-habituated Lactuca callus extracts. GC-MS(SIM) estimates were based on the base ions (m/z 130 and 132) as these were of greatest abundance.

Table 3.10 demonstrates a considerable difference between the levels of endogenous IAA in the callus tissues estimated by HPLC-F and GC-MS(SIM). This difference is

still evident after the HPLC-F results are corrected for overestimation. This would suggest further contamination of the methylated IAA peaks in the callus extracts shown in Figures 3.13 and 3.14.

The GC-MS(SIM) estimations do, however, reflect the same trend of a higher levels of IAA in the habituated tissue after a second separation step of GC, when the initial HPLC-F quantification of these levels were very similar in the two extracts (Table 3.5). These results clearly demonstrate that a further purification step would be required to obtain an extract of sufficient purity to quantify accurately and this step should ideally be based on a different separatory principle one.

The separation of the methylated plant extracts on HPLC-F (Figures 3.13 and 3.14) was based on the same reverse phase, isocratic separation and this was obviously not adequate for the separation af all contaminants. The contamination evident in the GC-MS traces of the plant samples demonstrates that even the use of the most advanced physicochemical techniques is not without problems if the instrument is not routinely available. The first GC-MS(SIM) run of a plant extract (Fig. 3.17) did not show any significant contamination after purification by the above technique as the second analysis, where contamination was very evident. The considerable difference in the levels of IAA quantified by the two techniques of HPLC-F and GC-MS(SIM) suggest that GC-MS should be used whenever possible but the instrument must be available for routine

analysis and the development of optimal conditions for the saparation of IAA on the GC. Alternatively, if HPLC must be used for quantification,then extracts should first be rigourously purified to ensure that they are of sufficient purity for accurate quantification.

3.7 Discussion

The results of the IAA analysis in the Lactuca callus tissues provide strong evidence for the existence of IAA as a natural endogenous auxin in both the habituated and non-habituated tissues. GC-MS was employed to verify the existence of IAA in the extracts quantified by HPLC-F and also in an attempt to validate the quantitative HPLC-F estimates obtained. GC-MS(SIM) analysis of the putative IAA isolated from the habituated tissue by the purification technique described in this Chapter produced an identical ratio of molecular to base ion abundance (31%) to that of standard methyl IAA at an identical retention time (Figures 3.16 and 3.17), a strong indication that IAA had been isolated from this tissue.

Persuasive evidence was also obtained for the presence of IAA in the non-habituated tissue in a further examination of plant tissue extracts on GC-MS(SIM). This second quantitative GC-MS(SIM) analysis provided molecular to base ion ratios of 32% and 33.5% at the retention time of authentic IAA in the habituated and non-habituated extracts respectively. These ratios were within the range obtained for standard methyl IAA in several preliminary

GC-MS(SIM) runs (Appendix G) but did not correlate well with the ratio obtained for standard methyl IAA on the same analytical run. This was the result of a contamination problem which also caused difficulties in the quantitative determination of IAA by GC-MS(SIM).

Difficulties were experienced in the estimation of IAA levels by both HPLC and GC-MS(SIM). The levels of IAA estimated by HPLC with a fluorescence detector gave poor correlation with levels quantified by GC-MS(SIM) in callus tissue extracts analysed at comparative stages of growth (Table 3.10). No direct comparison of IAA levels in plant tissues quantified by both HPLC-F and GC-MS(SIM) was, however, obtained due to the absence of appropriate internal standards at the time of analysis and thus no direct validation of the HPLC-F estimates of IAA was achieved.

The HPLC-F quantifications of IAA were, however, shown to be overestimates of the actual levels present in the tissues after derivatisation of the extracts. Methylation of the putative IAA recovered from both tissue extracts indicated the overestimation of IAA concentrations by at least 35% in the habituated tissue and 55% in the non-habituated one. This was estimated from the peak areas integrated on HPLC chromatograms which showed that these percentages of the derivatised extracts did not possess a similar retention time to authentic methyl IAA on a subsequent isocratic HPLC-F separation (Table 3.6).

Contamination of the putative IAA quantified by HPLC

was undoubtedly a major factor in the discrepancy observed between the levels of IAA estimated by the two physicochemical techniques. The difference in degree of contamination of the putative IAA isolated from the habituated and non-habituated extracts, demonstrated by derivatisation of the samples, was reflected in the levels of IAA measured by the GC-MS(SIM) technique. The habituated tissue contained a higher level of IAA at 7 days than the non-habituated culture contained at 14 days, after correction for overestimation, when the initial estimates were similar (Table 3.5).

Acknowledging the extent of contamination of the putative IAA initially quantified by HPLC, as indicated by derivatisation of the extracts, further contamination of the putative methyl IAA peaks in the plant extracts was suggested by the difference which remained between the corrected HPLC estimates and the levels quantified by GC-MS(SIM)(Table 3.10). The possibility exists that chromatographically similar carboxy or sulphoxy acids may have been present which readily methylated alongside IAA (Cohen 1984) but were not resolved by the second HPLC separation. A modified HPLC separation technique based on an alternative chromatographic principle or the simple introduction of gradient elution or an ion pairing agent (Sandberg et al 1981) might have separated further contaminants from authentic methyl IAA in the extract. The problem in the accurate estimation of the putative IAA recovered from the purification procedure would, with

hindsight, have been relieved by the addition of a second or more rigorous HPLC separation prior to quantification by HPLC-F.

The problems of obtaining a pure isolate of IAA from plant tissues for quantification are well documented (Sandberg et al 1987). An extensive range of chromatographic techniques have been employed in the purification of IAA from plant tissues (Sandberg et al 1987). These methods include PVP (Martin et al 1986, Sandberg 1981), Sephadex LH20 (Pengelly et al 1981,1986, Wyndale et al 1985, 1988), Sephadex G-25 (Sweetzer and Swartzfager 1978), DEAE Sephadex A-25 (anion exchange) (Sweetzer and Swartzfager 1978, Pengelly et al 1981, 1986) and TLC (Martin et al 1986, Iino et al 1980, Little et al 1978). These techniques are often laborious and time-consuming and may result in low recoveries of IAA from the purification (eg. Little et al 1978, Mann and Jaworski 1970).

Rigorous purification is not always necessary for low contaminant tissues (Akiyama et al 1983, Martin et al 1986). Akiyama et al (1983) reported a rapid and simple purification procedure for the isolation of IAA from radish seedlings. C18 Sep-Pak cartridges were employed to purify acidic indole extracts of the seedling tissue prior to a single isocratic HPLC separation from which IAA was quantified simultaneously by fluorescence and U.V. absorption. HPLC was carried out on a C18 reverse phase column, based on the same separation principle as the C18

Sep-Pak. The use of two subsequent chromatographic steps which use the same principle of separation provides limited clean up of the plant sample, where the Sep-Pak acts merely as a preparative clean up column for the HPLC (Sandberg et al 1987).

In this present work a single C18 Sep-Pak separation prior to HPLC was not found to be adequate for the isolation of a single peak on HPLC which co-incided with standard IAA. A second Sep-Pak cartridge (silica) based on an alternative normal phase separation was introduced to provide a combined Sep-Pak separation step capable of separating peaks from the tissue extracts on HPLC. The work of Akiyama et al (1983) was not validated by GC-MS and it is possible that the extracts quantified by HPLC-F were contaminated although the HPLC-F estimates correlated well with U.V. estimates obtained simultaneously and results of Avena straight growth bioassays also indicated that the majority of the extract quantified was IAA.

Ideally, every technique employed for the quantitative analysis of IAA or any other growth regulator should be validated prior to use by the most accurate method available. GC-MS is the only method available for the unequivocal identification of IAA but this technique is not without problems in the absence of an instrument of routine availability as the results of this present work illustrate.

Alternative preliminary methods of validating purification and quantification techniques for IAA and

other plant growth regulators include bioassays which quantify growth regulators on the basis of a growth response elicited in plant tissues by the isolated plant extract. Bioassays for auxin activity have low specificity for IAA as they quantify the total amount of all active auxins present in the plant extract. Bioassays have, however, been extensively employed in the capacity of validating IAA analyses obtained using physicochemical techniques (eg.Kutacek et al 1981, Akiyama et al 1983) and also as a sole method of quantification (Nakajima 1979).

When HPLC is used as for the quantification of IAA, selective fluorescence or electrochemical detectors are considered the detectors of choice due to the sensitivity and selectivity for IAA offered by these instruments (Sweetzer and Swartzfager 1978, Crozier et al 1980). A modified fluorescence detector can detect levels of IAA of 50pg and below (Crozier et al 1980, Sweetzer and Swartzfager 1978). Less specific detectors of IAA such as U.V. detectors may, however, be employed to help validate estimates of IAA obtained from other more specific detectors (eg. Akiyama et al 1983).

Reeve and Crozier (1980) have proposed the idea of successive approximation as a means of validating quantification techniques for plant growth regulators. This technique relies upon the use of successive methods of quantification for a plant extract until two or more of the IAA estimates obtained are in close agreement. Such rigorous validation need only be applied prior to the

routine analysis of plant extracts by a chosen technique.

Further problems were encountered in the analysis of IAA from Lactuca tissues in this work by the low level of recovery of IAA from the purification procedure (Table 3.4). Losses of the range and magnitude obtained in this work have been reported in the literature. Little et al (1978) recorded high losses of IAA, upto 99.5%, during the purification of IAA from cambium extracts of Picea sitchensis bark.

Poor recoveries of IAA demonstrate the great care which must be taken during the analysis of IAA regarding the purification steps chosen and the choice and purity of the reagents used. The use of redistilled solvents in addition to the anti-oxidant, sodium diethyldithio-carbamic acid, used would undoubtedly have reduced the losses incurred during purification (Chen et al 1988) as would a more extensive optimisation of each purification step.

Extreme care must be taken during the purification of IAA from plant tissues. In aqueous solution IAA is sensitive to acid, visible and U.V. light, ionising radiation, peroxide and peroxidases and this lability is enhanced by exposure to oxygen (Martin et al 1986). For this reason, it is preferable to lyopholise harvested tissues prior to analysis (Martin et al 1986) and avoid light and oxygen whenever possible throughout purification.

The extensive use of solvents, particularly those which have not been redistilled, inevitably contributes to the breakdown of IAA. Anti-oxidants such as sodium diethyl

dithiocarbamic acid (Ernsten et al 1986) or BHT (Iino et al 1980, 1982) are often introduced to minimise both the breakdown of IAA and various other indole interconversions which could seriously alter the amount of endogenous IAA quantified in the tissues as these chemicals have been shown to have a stabilising influence on indoles including IAA (Ernsten et al 1986). This stability is particularly important during the initial extraction of plant material in solvents as autolytic enzymes are released from plant tissues by homogenisation (Yokota et al 1983).

The results of this present work showed that IAA was lost at all stages of the purification procedure similar to the observations of Little et al (1978) and Mann and Jaworski (1970) who also suffered high losses of IAA during purification. This suggested a general level of IAA loss in addition to the specific losses of IAA incurred at each purification step. This inevitably includes periods of storage of partially purified extracts and the use of solvents which were not redistilled.

The use of 5-^{3}(H)IAA as an internal standard would not be recommended on the basis of the problems experienced in the loss of purity of this labelled compound over a short period in storage. This lability undoubtedly contributed to the introduction of some degree of error in quantification. Tritiated IAA is, however, still used extensively as an internal standard for the quantitative estimation of endogenous IAA levels in plant tissues (eg. Pengelly et al 1986) because of the high specific activity of the

compound.

The HPLC-F estimates of endogenous IAA levels in the Lactuca callus tissues were within the physiological range expected for actively dividing plant tissues (ie. 1-100ng/g fwt (Sweetzer and Swartzfager 1978)) and disorganised plant tissue cultures. The levels of IAA reported in cultured tissues range from undetectable to above 1 µg/g fresh weight (eg. Kevers etal 1982).

Acknowledging that the levels of IAA quantified in the Lactuca tissues were overestimates, the levels of IAA determined in the habituated and non-habituated tissues may still be compared. The corrected HPLC-F estimates demonstrate that the habituated tissue contained an elevated concentration of IAA at all three stages analysed during the culture period (Table 3.7).

The habituated tissue was of a slightly higher growth rate (4 day doubling time compared with 5 days in the non habituated tissue) and this factor may have had some bearing on the results obtained. Koves and Szabo (1987) observed a difference in IAA levels quantified in habituated and non-habituated tissues of tobacco and thought this to be related to a difference in growth rate between the two cultures.

The IAA estimates for the Lactuca tissues support the suggestion that elevated levels of endogenous growth regulators may exist habituated tissues and may cause the autonomous growth observed in these cultures. There is evidence that increased levels of endogenous IAA exist in

certain other auxin-independent tissue cultures of crown gall origin (eg. Mousedale 1982). Kulescha and Gautheret (1948) measured higher levels of extractable auxin in an auxin-independent Scorzonera hispanica tissue culture than in the auxin-dependent tissues from which the culture was originally derived.

Meins (1982) has postulated that the production of growth factors by habituated cultures allows these tissues to proliferate without an exogenous supply of these substances. This idea was supported by the observation that cytokinin-habituated tobacco Havana 425 cultures, when subjected to a cold treatment of 16°C, were seen to lose their cytokinin-independence. It was proposed that the production of cell division factors in the tissue had been halted by the cold treatment, thus preventing the growth of cultures in the absence of additional cytokinin (Binns and Meins 1973).

However, most recent evidence concerning the levels of endogenous IAA in auxin-independent tissue cultures, including habituated tissues, does not support the theory that elevated levels of growth regulators exist in these autonomous tissues. Nakajima et al (1979) was unable to detect either auxin or cytokinin in three seperate habituated cell suspension cultures of tobacco. This may, in part, have been due to the use of bioassays as the method of detection and endogenous growth regulators may have been detectable using more advanced physicochemical techniques. Wyndaele et al (1988) also found levels of IAA

in a habituated soybean line to be very low whilst in another habituated culture the levels were elevated over a control tissue concentration.

Kevers et al (1982) monitored extremely high levels (>1μg/g fwt.) of endogenous IAA in sugarbeet calli using gas chromatography with an electrochemical detector but found that the levels present were equivalent in both a habituated and a non-habituated line. Kövesand Szabo (1987) and Kutacek et al (1981) employed the 2-MIP assay for the quantification of IAA in habituated and non-habituated tissues of tobacco. Differences in IAA levels observed between the habituated and non-habituated cultures analysed by Koves and Szabo (1987) were attributed to the difference in growth rate of the two tissues and not to habituation. Kutacek et al (1981) reported almost equivalent concentrations of IAA in habituated (128 ng/g fwt.) and non-habituated (149 ng/g fwt.) cultures analysed at a single stage of growth.

These reports suggest that a direct correlation between auxin-habituation and endogenous IAA levels does not exist in all auxin-independent tissues. In support of this idea are the results of Wyndaele et al (1988) which demonstrated that two separate lines of auxin-habituated soybean tissue had drastically different levels of endogenous IAA, one elevated and one lower than a control tissue.

A prominant peak in IAA levels was observed during the 21 day culture period of the Lactuca tissues analysed in

this work. This peak in IAA concentration, with levels varying upto 100 fold during the culture period, was measured later in the non-habituated tissue than in the habituated culture and this may have been related to the comparatively slower growth rate of the non-habituated tissue. No growth data was obtained for the growth cycle of the tissues from which the IAA was extracted for these estimates and therefore no direct correlation between IAA concentration and the stage of growth of the tissues was possible.

Dramatic fluctuations in IAA levels during the growth cycle of cultured plant tissues have been observed in several other tissue cultures. These peaks in IAA concentration may be correlated with a specific stage of growth. Moloney et al (1983) observed a peak in IAA levels, with levels varying by as much as 10 fold, approximately 7 days after transfer of an auxin-dependent Acer cell suspension to fresh medium. The highest levels of IAA measured in this culture were found to be correlated with the period of intensive growth in the tissue. Similarly, the very high concentrations of IAA quantified by Kevers et al (1982) in sugarbeet tissues were measured during the period of exponential growth.

Prominant peaks in IAA concentration have also been recorded in auxin-independent gall tissues of A. tumefaciens (Pengelly et al 1986) and Zizyphus (Tandon and Arya 1980). Pengelly et al (1986) analysed IAA in four crown gall cultures by RIA and observed a peak in IAA

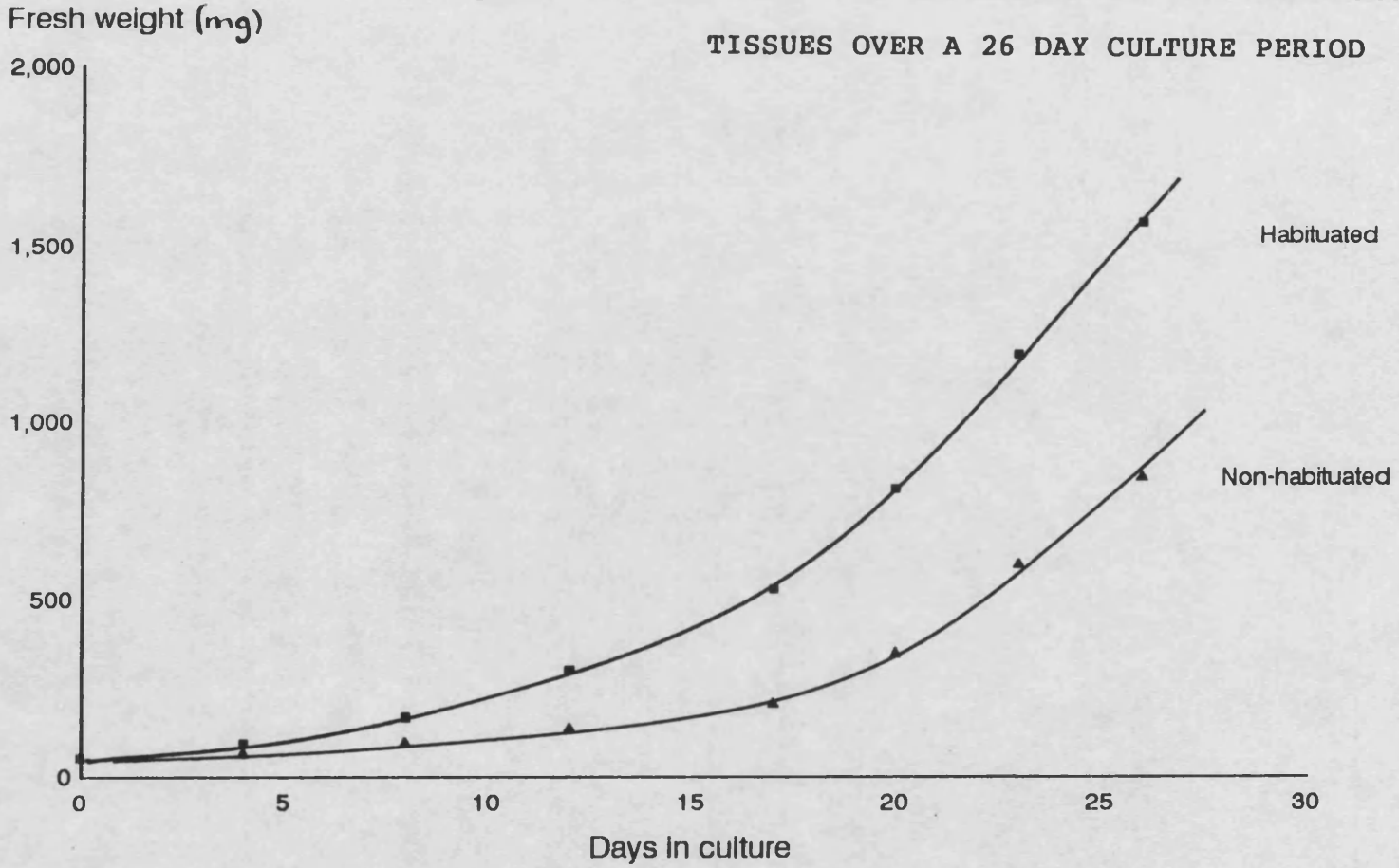
levels at 6 days after subculture (analysis was carried out at 6, 14 and 23 days) in 3/4 of these tissues. The concentration of IAA in the galls was seen to fluctuate approximately 10 fold during the 23 days. In the Zizyphus gall tissue, Tandon and Arya (1980) analysed IAA by paper chromatography at five day intervals throughout a 30 day culture period. Levels of IAA were of the same order of magnitude throughout the culture period but a peak in IAA concentration was observed at day 10. In contrast to these observations, Atsumi (1980) and Köves and Szabo(1987) detected highest levels of IAA at the onset of the stationary phase in crown gall (Atsumi 1980) and normal and habituated tissues of tobacco (Köves and Szabo 1987).

The often contradictory evidence concerning the role of endogenous IAA levels in the maintenance of auxin-independent growth demonstrates the need for more truly comparative routine estimations of IAA in habituated and non-habituated plant tissues using the most advanced physocochemical techniques available, such as GC-MS(SIM). An extensive study of the fate of IAA levels during a culture cycle is also necessary before any conclusions may be drawn concerning the role of IAA and IAA levels in the regulation of cell division and growth in plant tissues.

Fig. 3.23 : The growth characteristics of the habituated and non-habituated tissues over a period of 26 days. The non-habituated tissue demonstrates a longer lag phase and overall slower growth rate than the habituated tissue.

Fig. 3.24 : Comparison of the growth characteristics of the habituated and non-habituated tissues over a period of 26 days. The non-habituated tissue demonstrates a biphasic pattern of growth with a lower growth rate than the habituated tissue for the first 15 days followed by a rapid burst of growth at a rate equivalent to that of the habituated tissue. The habituated tissue shows a consistently high growth rate over the 26 day culture period.

Fig. 3.23 : GROWTH OF THE HABITUATED AND NON-HABITUATED TISSUES OVER A 26 DAY CULTURE PERIOD



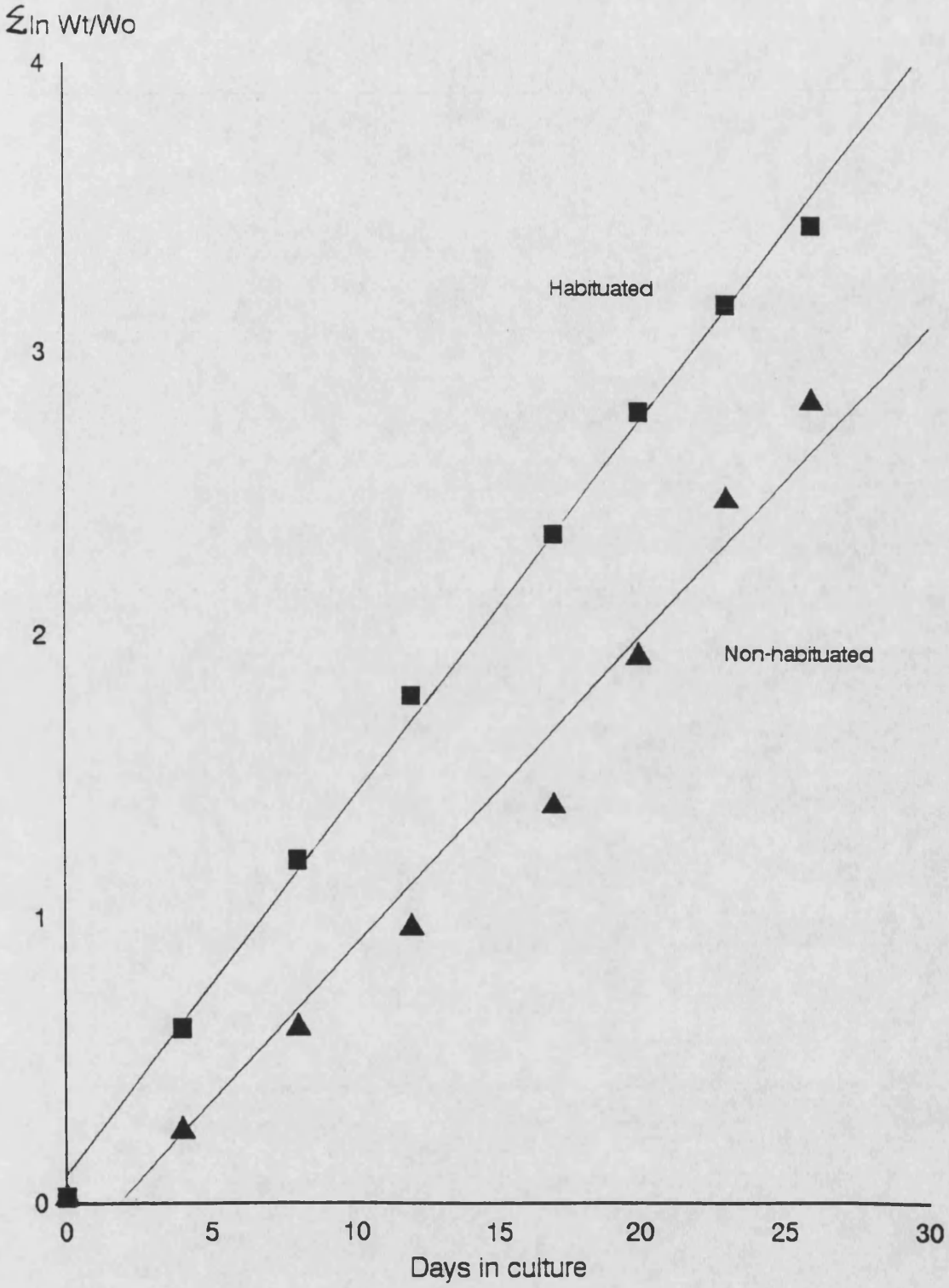


Fig. 3.24 : GROWTH OF THE HABITUATED AND NON-HABITUATED TISSUES OVER A 26 DAY CULTURE PERIOD

Chapter 4

Studies on IAA metabolism in Lactuca callus tissues

4.1 Introduction

The results of Chapter 3 indicate that the levels of endogenous IAA are important to the expression of the habituated phenotype. This suggests that alterations have taken place in the regulation of IAA metabolism in habituated tissues. However, aberrant regulation of IAA metabolism will not necessarily cause an alteration in the levels of endogenous IAA, but might merely involve a shift in the homeostatic control mechanism by which these levels are maintained. For this reason, it was decided to compare the metabolism of IAA in auxin-dependent and independent cultured plant tissues. Endogenous levels of IAA may be regulated by one or a combination of three pathways of IAA metabolism in plant tissues: (1) biosynthesis, (2) conjugation and (3) degradation. The aim of the following work was to investigate IAA metabolism in Lactuca callus tissues.

Firstly, the metabolism of ^{14}C tryptophan was followed in both habituated and non-habituated callus tissues with the aim of identifying the extent of de novo IAA synthesis occurring in these cultures and to provide an indication of the pathways involved. Secondly, an attempt was made to determine the extent of IAA conjugation in the non-habituated tissue, which responded to culture in the dark with a significant reduction in growth rate. Bandurski et al (1977) have proposed that the formation and hydrolysis

of IAA conjugates could function as a homeostatic control mechanism for the regulation of IAA levels in plant tissues.

4.2 Materials and methods

Chemicals

All standard indoles used in TLC analysis (indole-3-acetic acid, indole-3-pyruvic acid, indole-3-acetaldehyde, indole-3-acetamide, indole-3-acetonitrile, tryptophan, tryptophol and tryptamine) were purchased from Sigma Chemical Company. All solvents used were of analytical grade and were obtained from B.D.H., Poole, England. The chemicals used in auxin analysis were as described in Chapter 3.

Plant material

Habituated and non-habituated Lactuca callus tissues were cultured on Murashige and Skoog (1962) medium with supplements of NAA (1 mg/l) and kinetin (0.5 mg/l) for the non-habituated tissue.

4.2.1 Analysis of IAA biosynthesis in habituated and non-habituated Lactuca tissues

Procedure for feeding ^{14}C tryptophan to callus tissues

Tissues were harvested for ^{14}C tryptophan feeding studies 10 days after subculture. Five gram pieces of callus tissue were weighed, aseptically into 10 x 1cm sterile plastic petri dishes to which 10ml of liquid medium

(Murashige and Skoog (1962), 2% sucrose) was added. The feeding solution for the non-habituated tissue was supplemented with NAA (1mg/l) and kinetin (0.5 mg/l). The callus tissues were gently teased apart in the feeding solution using a pair of sterile forceps. 0.5 ml of L-methylene ^{14}C tryptophan (74 KBq, 1.85-2.22 GBq/mmol) was added to each culture through a 0.2μ sterile filter (Gelman Sciences). The dishes were then sealed with a thin strip of parafilm and incubated at 25°C in continuous light (685 μ mols m s) on a rotary platform (65 rpm) for 40 hours.

Preparation of extracts for TLC

Extracts of indole derivatives were prepared from the tissues for TLC analysis using a solvent extraction technique described by Lui et al (1978). After incubation with ^{14}C tryptophan, the cultures were filtered on Whatman No.1 discs under suction. The filtrate was removed and stored at 4°C. The tissue on the filter was rinsed with 15-20 ml of ice-cold 0.1 M phosphate buffer (pH 7) before being carefully removed from the filter with a small spatula and placed in a glass homogeniser tube. 10 ml of the phosphate buffer was added and the tissue homogenised. The homogenate was made up to a final volume of 100ml with distilled water. The pH was adjusted to 11.0 by the addition of 1M NaOH. The extract was then partitioned twice against an equal volume of diethyl ether in a glass stoppered bottle using magnetic stirring. The organic extracts were removed, pooled and evaporated to dryness in

a 250 ml flask at 40°C in vacuo. The sample was then taken up in 5ml of methanol and transferred to a 5 ml glass centrifuge tube using a pasteur pipette. The contents were evaporated to dryness in a centrifugal freeze dryer. The extract was finally taken up in 1 ml of methanol and stored at -20°C prior to TLC separation.

The aqueous extract of pH 11 was then neutralised to pH 7 by the addition of 2 M HCl and the above procedure of partitioning against diethyl ether was repeated with the ether extracts evaporated to dryness and taken up in 1ml of methanol. A final acidification of the aqueous extract to pH 3 with 2 M HCl was followed by partitioning the sample against ether and this extract was also treated as above. The incubation medium was stored at -20°C until required, when it was removed from the cold and its pH adjusted to 11, 7 and 3. Ethereal extracts were prepared at each pH as described above for the tissue extracts. A total of six extracts were obtained for each callus type. All extracts were stored at -20°C prior to TLC separation.

TLC separation

The tissue and medium extracts were applied in 10µl aliquots to solvent-compatible wicks presoaked in solvent from underlying pools set in a radial TLC apparatus. The TLC apparatus used is illustrated in Figure 4.1. A silica gel TLC plate (20 x 20 cm, Schneider and Schnell, West Germany) was placed on top of the wicks and the samples, four per block, were run for approximately 30 minutes until

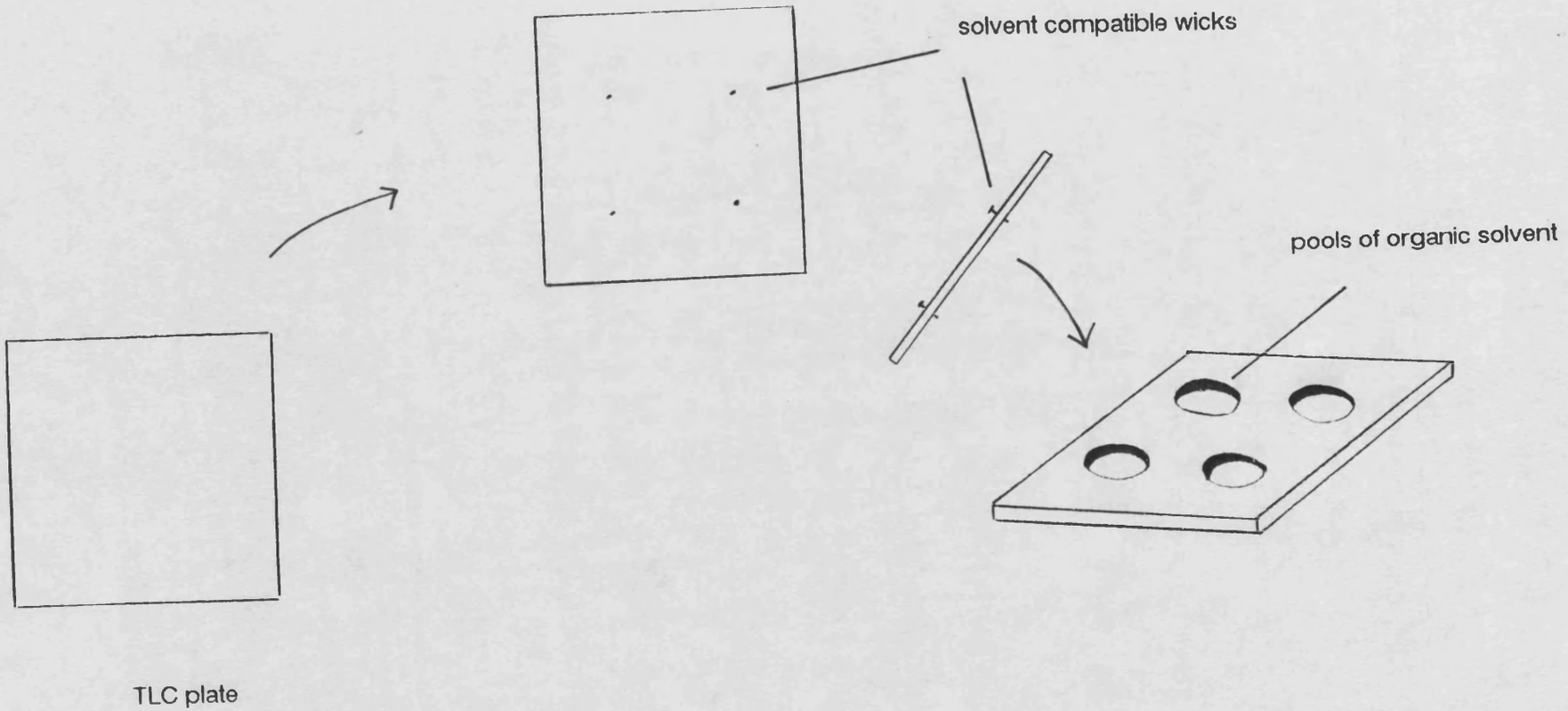


Figure 4.1 : Radial TLC apparatus used for the separation of indoles.

a ring of 8 cm diameter was formed by the solvent front. The solvent systems used were (1) a butan-1-ol: ethanol: 25% ammonia mixture (8:1:1 by volume) and (2) a chloroform: glacial acetic acid mixture (95:5 by volume). This latter solvent was employed in an attempt to separate the non-ionisable indoles which ran with the solvent front in the first solvent system. The TLC plates were dried with the aid of a hair dryer after marking of the solvent fronts. The distance travelled by standard indole compounds were then determined by viewing the plates under U.V. light at 380 nm and marking the position of the dark rings formed by indole absorption of the U.V. light. Several standard indole compounds were tested and the Rf values obtained for these compounds in the two solvent systems are presented in Table 4.1 (page 167).

For the analysis of radiolabelled metabolites of ^{14}C tryptophan in the tissue and medium extracts, dried TLC plates, with solvent fronts marked, were placed in contact with X-ray film (Fuji, Japan) in complete darkness. The plates and film were secured into autoradiography cassettes and left at -20°C for four weeks, prior to development of the film.

Due to limited apparatus and time, only four TLC plates could be analysed, limiting the number of extracts which could be run in both solvent systems. Only non-ionisable indole standards were not separated using solvent system (1) (Table 4.1). These compounds ran with the solvent front. For this reason, only extracts of pH 7 were

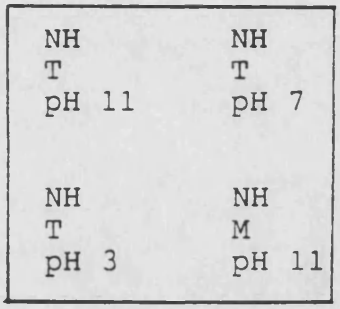


PLATE 1 Solvent 1

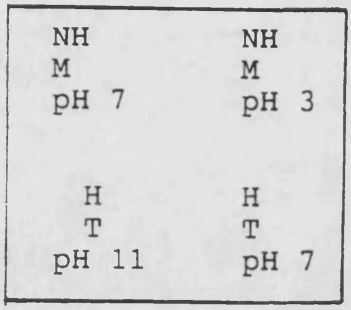


PLATE 2 Solvent 1

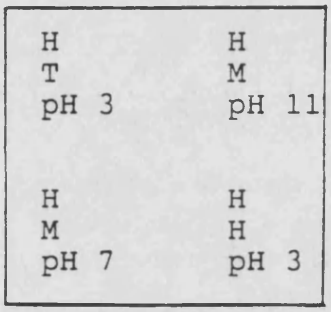


PLATE 3 Solvent 1

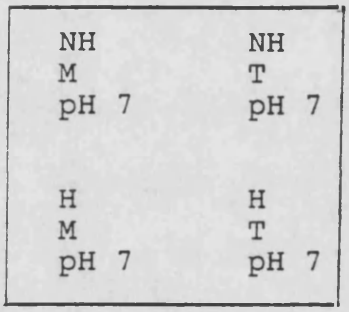


PLATE 4 Solvent 2

Solvent 1: Butan-1-ol: ethanol: 25% ammonia (8:1:1 by volume)

Solvent 2: Chloroform: glacial acetic acid (95:5)

T = Tissue extract M = Medium extract

H = Habituated extract NH = Non-habituated extract

Figure 4.2 : Loading pattern of tissue and medium extracts on TLC plates.

separated using both solvent systems as ionisable compounds could be adequately separated in solvent system (1). Non-ionisable compounds should only have been present in the neutral extract. The loading pattern of the extracts on TLC plates is illustrated in Figure 4.2.

The detection limit of the TLC-autoradiography technique used

The limit of detection of TLC-autoradiography for standard ^{14}C tryptophan was determined by the application of a range of ^{14}C tryptophan quantities (0.1ng to 100ng) to TLC plates which were then eluted in solvent (1), butan-1-ol:ethanol: 25% ammonia mixture (8:1:1 by volume). The TLC plates were dried then placed in contact with X-ray film and sealed into autoradiography cassettes. The cassettes were stored at -20°C for four weeks before development of the film. Visual inspection of the developed autoradiograms has shown that all of quantities of ^{14}C tryptophan applied were detectable. The detection limit of standard ^{14}C tryptophan was therefore below 0.1ng.

4.2.2 Estimation of free and conjugated IAA levels in callus tissues in response to culture in the dark

Estimation of levels of free and bound IAA in Lactuca callus tissues

Bandurski et al (1977) have proposed that the formation and hydrolysis of IAA conjugates may be a regulating factor in the levels of endogenous IAA in plant tissues. Evidence for this hypothesis was provided by the observation that a significant increase in free IAA levels, accompanied by a decrease in conjugate forms of the auxin, occurred in Zea mays seedlings in response to a growth-inhibitory light flash.

There was preliminary evidence to suggest that culture in the dark was non-permissive for the habituated Lactuca tissues used in this present work. This habituated tissue was originally isolated by Savigear (1985) and has been maintained on a medium free of growth regulators for more than 4 years. An observation was made by Ward (1985) that the growth of this tissue was completely inhibited by culturing in the dark for a six week period. An investigation of the effect of culturing both the habituated and non-habituated tissues in the dark was therefore carried out to determine the ability of these tissues to grow in the absence of light. This was an attempt to determine whether light-induced changes in growth were observed in the tissues and whether the levels of free and bound IAA were altered with any alterations in growth.

Determination of growth rate of Lactuca callus tissues grown in the presence and absence of light

Habituated and non-habituated tissues were used in this experiment and were subcultured from stock material isolated and maintained as described in Chapter 2. Cultures were grown on Murashige and Skoog (1962) medium (pH 5.5, 0.7% agar, 2% sucrose) supplemented with NAA (1 mg/l) and kinetin (0.5 mg/l) for the non-habituated tissue. The cultures were incubated at 25°C.

For cultures to be grown in the dark, the culture jars were covered with a double layer of aluminium foil. When these cultures were subcultured after 3 weeks the only permissible light source in a completely darkened room was the light of the laminar air flow cabinet which was covered with a dark blue filter ("Masterline Cinemoid 19 A"). The wavelength transmission of this filter is presented in Figure 4.3 and demonstrates the low or 0% transmission between the wavelengths of 550-750nm. Light from the flame and the weighing balance were shielded from the tissues by a layer of aluminium foil.

Growth rates for both the habituated and non-habituated tissues were determined by fresh weight analysis after 3 and 6 weeks in culture. The doubling constant, k, was then calculated for each culture and growth curves were obtained for the Lactuca tissues grown in the light and in the dark. These are presented in Figures 4.4 and 4.5.

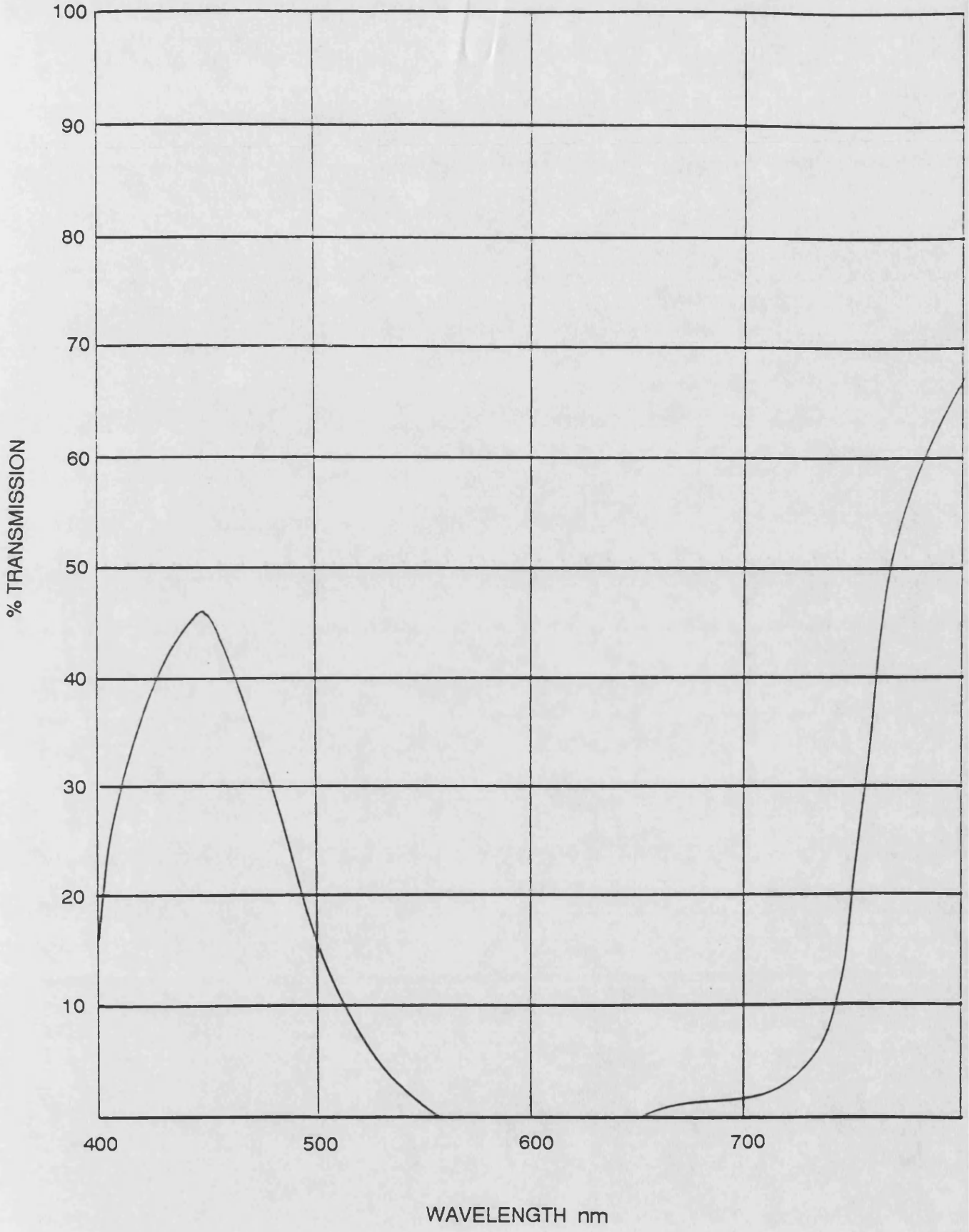


Figure 4.3 : Wavelength transmission of 'Masterline Cinemoid 19A' dark blue filter.

Figure 4.4 demonstrates that the habituated Lactuca tissue was not inhibited by culturing in the dark for six weeks. In fact, the growth of this culture was slightly faster in the dark. This contrasts with the previous observation of Ward (1985) which indicated that light was an obligate factor for the growth of this tissue. The non-habituated tissue did, however, demonstrate a reduction in growth rate over a six week period of culture in the dark. The growth rate in the dark-grown tissue was 25% lower than the light-grown tissue.

The possibility that this observed reduction in growth rate was accompanied by an alteration in the levels of free and bound IAA in the tissue was examined by analysing the total and free IAA concentration in light and dark grown non-habituated tissues after 6 weeks in culture.

Estimation of free IAA

The levels of free IAA in both light and dark grown non-habituated tissues were estimated by the method developed in Chapter 3. A summary diagram of the purification procedure is presented in Figure 4.6.

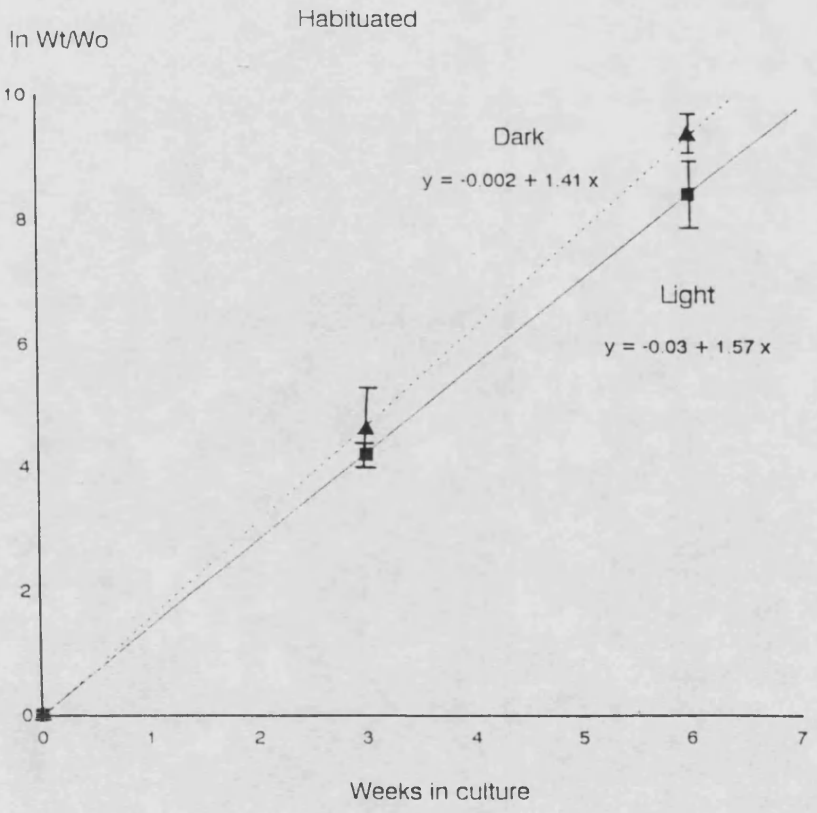


Figure 4.4 :

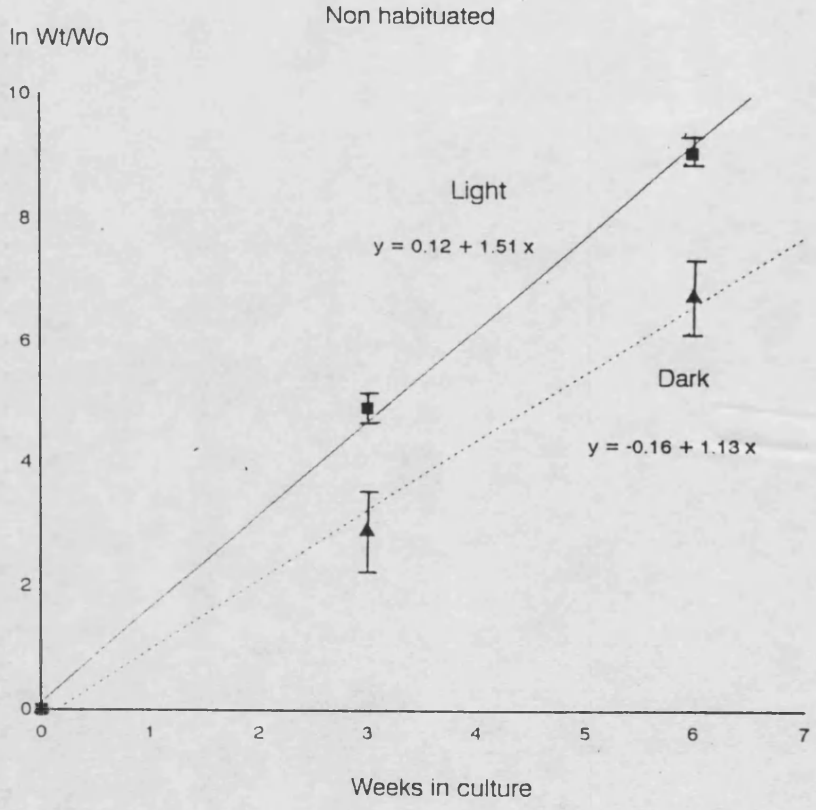


Figure 4.5 :

Figures 4.4 and 4.5 : Growth of habituated and non-habituated tissues in the light and dark. Error bars represent standard error of the mean.

Estimation of total (Alkali labile and free) IAA

The levels of conjugated (esterified) IAA in these tissues were estimated by determining the total amount of IAA (ie. free IAA and IAA released from mild hydrolysis in 1N NaOH). Total IAA was extracted and purified by the following method. A methanol extract was obtained from 2g of Lactuca callus tissue to which approximately 2.5 x 10^6 dpms of 5-^{3}(H)IAA were added as an internal standard. The extract was reduced to the aqueous phase in vacuo and then made alkaline by the addition of 1N NaOH. The extract was left at room temperature (22-25°C) for 1 hour. This alkaline hydrolysis was employed by Bandurski and Schulze (1974, 1977) to release esterified IAA from Zea mays seeds and seedlings.

The hydrolysed extract containing free IAA and IAA released from alkaline hydrolysis was acidified to pH 8 by the addition of HCl, in the presence of 0.1 M phosphate buffer (pH 8). Further purification of the hydrolysed extract (total IAA) was by the method described for free IAA (Figure 4.6). Peaks on the HPLC profiles which were co-incident with the retention time of IAA were collected and aliquots removed for the determination of 5-^{3}(H)IAA recovery. Endogenous IAA levels, total and free, were quantified by the isotope dilution method (Rittenberg and Foster 1940), which has been employed to quantify IAA released from auxin conjugates (Bandurski and Schulze 1977).

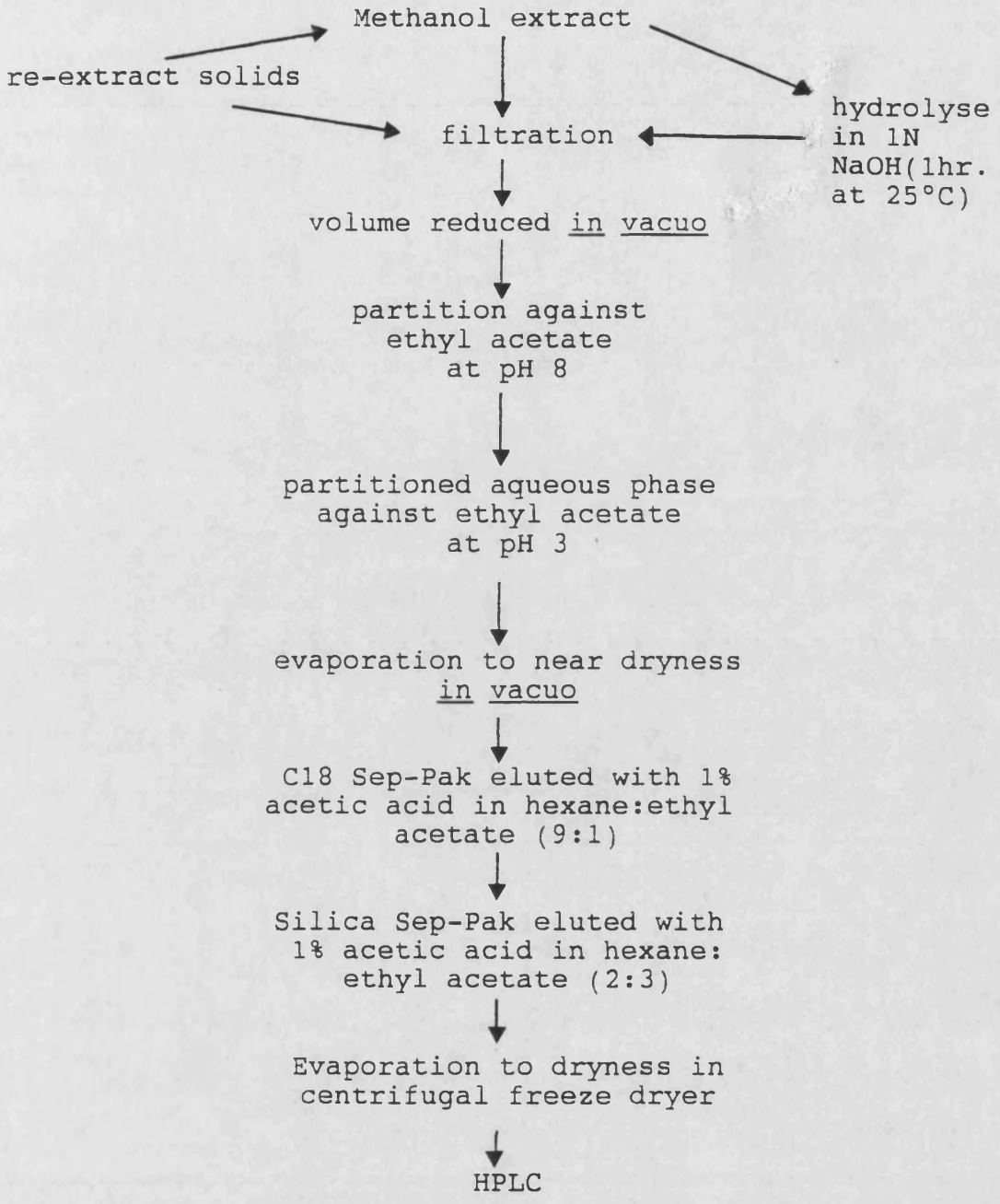


Figure 4.6 : Summary of the purification method for the estimation of free and bound IAA levels in Lactuca callus tissue.

4.3.1 Results of TLC analysis of ^{14}C tryptophan metabolites in Lactuca callus tissues

A single ^{14}C tryptophan feeding experiment was performed in both the habituated and non-habituated Lactuca callus tissues. The Rf values of ^{14}C tryptophan metabolites recovered from the tissues were determined by division of the distance travelled by the compound relative to the solvent front. A limited number of ^{14}C metabolites of tryptophan were recovered from the tissue extracts of both culture types, demonstrating a low recovery of initial radioactivity. Only three or four compounds of distinct Rf values were obtained (Table 4.2) from the tissues and the amount of each metabolite recovered was approximately equal to or less than 0.1ng, determined by a visual comparison of the autoradiograms with that of a range of ^{14}C tryptophan standards. This would suggest that approximately 10ng of each metabolite was present in the initial tissue extracts, representing 5g fresh weight of callus. This would, in turn, mean that only approximately 30-40 ng of metabolites were recovered from the 7 µg of tryptophan fed to the tissues, about 1%.

No ^{14}C tryptophan metabolites were recovered in the medium extracts indicating that either no extracellular conversions of tryptophan took place or that such conversions were below the detection limit of this TLC autoradiography technique (ie.< 0.1 ng of product produced).

The pattern of ^{14}C metabolites of tryptophan produced

in the Lactuca tissues was very similar in both tissues. No compound with an Rf value close to that of IAA was recovered from either tissue.

Standard indole derivative	Rf in solvent 1	Rf in solvent 2
tryptophan	0.37	0.1
tryptophol	1.0	0.63
indole-3-acetamide	0.9	0.55
indole-3-acetonitrile	1.0	0.7
indole-3-acetaldehyde	1.0	0.72
indole-3-acetic acid	0.53	0.68
indole-3-pyruvic acid	0.1	0.82
tryptamine	0.42	0.13

Table 4.1: Rf values for standard indoles run in two solvent systems.

(1) Butan-1-ol : ethanol : 25% ammonia (8:1:1).

(2) Chloroform: glacial acetic acid (95:5).

All standards were applied as solutions of 10^{-3}M.

No tryptophan metabolites were recovered in medium extracts of pH 3, 7 or 11. This meant that the uptake of tryptophan from the medium was near quantitative. The absence of ^{14}C IAA from the tissue extracts made the interpretation of the results difficult with respect to the study of IAA biosynthesis and suggested that either the level of IAA biosynthesis in the tissues was very low (ie. less than 0.1ng of tryptophan converted to IAA) or that IAA and other labile indole metabolites of ^{14}C tryptophan were lost during the purification and subsequent TLC separation.

	Tissue type					
	Habituated			Non-habituated		
pH of extract	3	7	11	3	7	11
Rf in solvent 1 Tissue						
Rf in solvent 2 Tissue	0.16	0.41	0.44	0.16 0.34	0.41	0.41
Rf in solvent 1 Medium						
Rf in solvent 2						

Table 4.2 : Rf values obtained from TLC autoradiography analysis of tissue and medium extracts of habituated and non-habituated Lactuca callus tissues.

There was also the conspicuous absence of a ^{14}C labelled compound with an identical or very similar Rf value to that of standard tryptophan. A compound of a similar Rf($\pm$ 0.03) was recovered in the acidic extract of the non habituated tissue. Liu et al (1978) has reported that tryptophan was often observed in acidic indole extracts prepared from tobacco callus tissues by this purification method. If this compound were tryptophan, the presence of non-metabolised tryptophan only in the non-habituated extract might suggest a lower level of tryptophan metabolism in this tissue. This could be related to the slightly slower growth rate of this tissue.

At least 50% of the recovered radioactivity from both tissues was attributable to a compound of Rf 0.41, also close to that of tryptophan ($\pm$ 0.04) but closer to that of standard tryptamine ($\pm$ 0.01). This compound was observed in both neutral extracts and the alkaline extract of the habituated tissue. A similar Rf value (0.44) was also recovered in the alkaline extract of the non-habituated tissue. The possibility that tryptamine was recovered from the tissues suggests that the is very interesting as this would suggest that the tissues utilised a biosynthetic pathway reperted in a limited number of species (Schneider and Wightman 1974).

The preliminary identification of the compound recovered in acidic extracts with an Rf of 0.16 was not possible from this analysis as the Rf value for this compound was not similar enough to that obtained for either

of the standard acidic indoles tested (ie.IPyA and IAA). This compound may, however, have been a breakdown product of IAA. Further identification of any of the indole metabolites recovered would require further characterisation. Spraying the plates with Erlich's reagent may have helped to distinguish between different compounds in the extracts in this preliminary identification but was not carried out on this occasion.

4.3.2 Results of HPLC-F estimates of free and total(free and bound) IAA

A 50% reduction in the levels of free IAA was observed in non-habituated Lactuca tissues cultured in the dark for a six week period (Table 4.3). This reduction was accompanied by a decrease in growth rate over this period. No significant difference in the level of total (free and bound) IAA was, however, observed mainly as a result of wide variation in the estimates obtained (Table 4.3).

IAA levels were estimated using HPLC with fluorescence for detection, after an initial purification by the method outlined in Figure 4.6. The hydrolysed extracts received no additional purification and the chromatograms of these extracts showed that further purification was necessary (eg.Figure 4.7). Most of the HPLC profiles showed peaks in

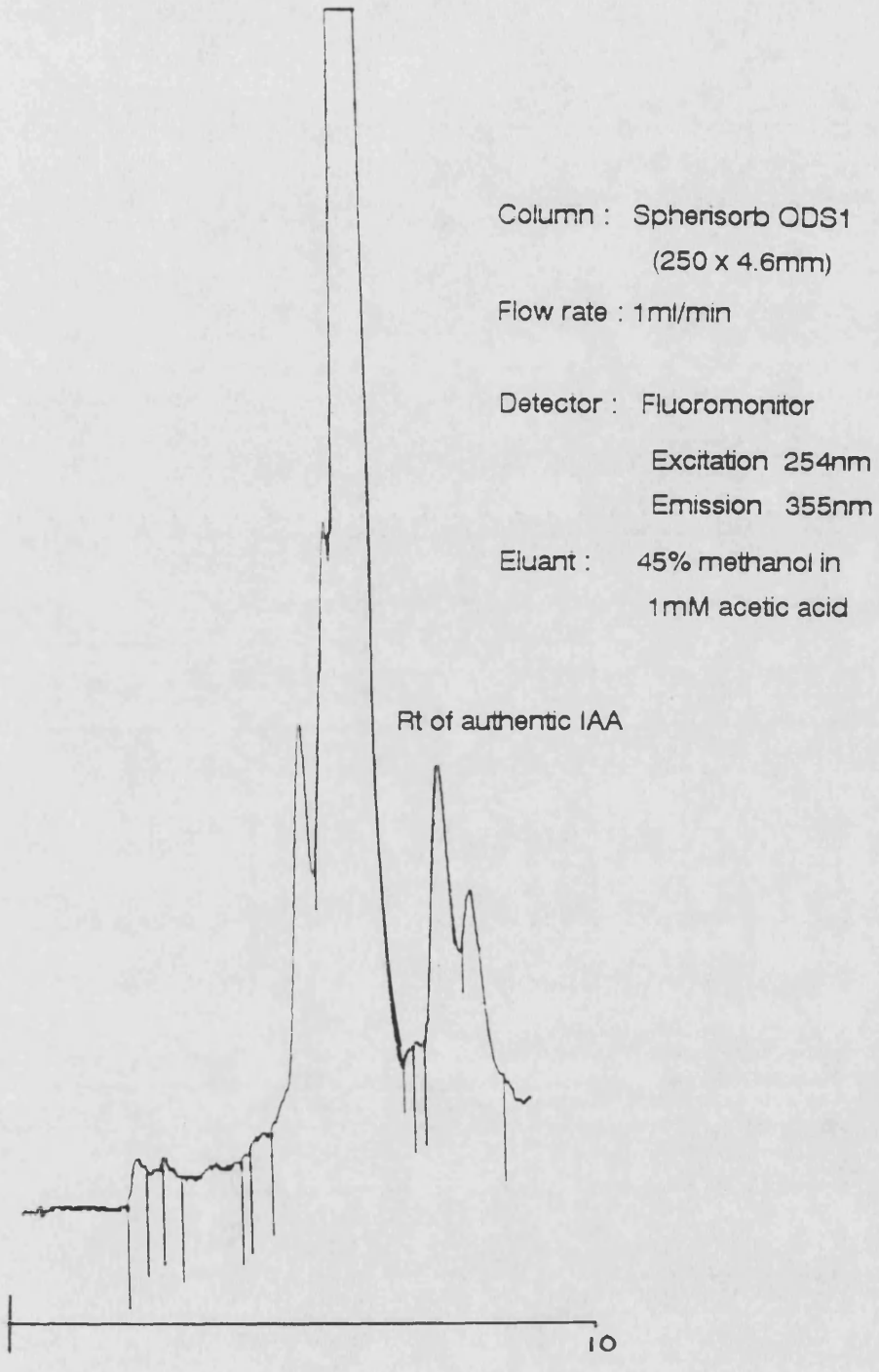


Figure 4.7 : HPLC chromatogram of a hydrolysed extract of non-habituated tissue.

the hydrolysed extracts which were visibly contaminated and there was a general low recovery of material in these extracts. No further characterisation of the putative IAA peaks from hydrolysed extracts were possible due to this low recovery of material and the extent of contamination of these peaks was therefor not established.

HPLC-F estimates of free IAA obtained using the technique described in this work are acknowledged overestimates (see Chapter 3) but may be appropriately reduced by the correction factor calculated in Chapter 3 (reduction of integrated areas by 54%) for the non-habituated extracts to obtain a more realistic estimate. The lack of further characterisation of the hydrolysed extracts prevented the comparison of free and total (free and bound) IAA as the degree of contamination of the putative IAA peaks was certainly not same in the two types of extract.

The poor recovery of material, including IAA, from the purification of the hydrolysed extracts was interesting as more, rather than less, material would be expected as a result of alkaline hydrolysis of the tissue extract. The hydrolysed extracts were observed to be highly pigmented. Pigmentation of non-hydrolysed extracts was typically removed during the solvent partitioning step of the purification. In the hydrolysed extracts significant pigmentation remained after partitioning against ethyl

Sample		Free IAA levels ng/g fresh weight	Free and bound IAA levels ng/g fresh weight
Light			
Replicate	1	13.2	186
	2	14.5	90
	3	6.8	/
	mean	11.5 ± 2.4	138 ± 48
Dark			
Replicate	1	3.1	254
	2	5.7	584
	3	8.7	/
	mean	5.8 ± 1.6	419 ± 165

Table 4.3 : HPLC-F estimates of IAA levels in light and dark grown non-habituated Lactuca callus tissues (values are corrected for known overestimation of 54%). Mean values are presented with standard error of the mean.

acetate. The poor recovery of material, including IAA, from the purification of the hydrolysed extracts was interesting as more, rather than less, material would be expected as a result of alkaline hydrolysis of the tissue extract. The hydrolysed extracts were observed to be highly pigmented. Pigmentation of non-hydrolysed extracts was typically removed during the solvent partitioning step of the purification. In the hydrolysed extracts significant pigmentation remained after partitioning against ethyl acetate. This pigmentation undoubtedly contributed to problems in the further purification of the samples, in particular the Sep-Pak cartridges which certainly work less efficiently as a result of overloading with excess material. Additional purification of hydrolysed extracts prior to the use of Sep-Pak cartridges would be necessary to obtain extracts of sufficiently reduced sample weight to be adequately purified by these cartridges and allow the subsequent separation of distinct peaks on HPLC-F. The analysis of esterified IAA does not necessarily require more purification than that applied to non-hydrolysed extracts (Bandurski and Schulze 1974, 1977) when rigorous purification steps are employed.

4.4 Discussion

The putative pathways of IAA biosynthesis from tryptophan in plant tissues have been identified using a combination of radiolabelled precursor feeding studies and enzyme assays involving either crude or purified cell-free tissue preparations (Schneider and Wightman 1974). The accurate analysis of the *in vivo* metabolism of IAA is difficult due to the inherently labile nature of the indole metabolites thought to be involved. These intermediates readily undergo chemical interconversions which can complicate the determination of the biosynthetic pathways involved (Reinecke and Bandurski 1987).

The low recovery of ^{14}C metabolites of tryptophan in this present analysis suggested that despite the possibly considerable loss of indole metabolites during the extraction and purification technique, the majority of tryptophan (a maximum of 99% in the absence of loss) had been metabolised in pathways other than IAA biosynthesis. Natural endogenous levels of tryptophan are reported to be at least one thousand fold higher than the levels of IAA (Epstein et al 1980). Not all of this tryptophan appears to be available for the synthesis of IAA. The sequestration of tryptophan away from the cytosol may be a mechanism by which the level of IAA biosynthesis is regulated from the amino acid precursor in the cells. Allen and Barker (1980) and Rekoslavskaya et al (1986) have reported that an inverse relationship exists between the levels of endogenous tryptophan and IAA in *Ricinus* leaves and tobacco

cell cultures respectively, suggesting that a quantitative conversion from tryptophan does not take place. These observations are in contrast to the those of Sung (1979), who measured elevated levels of both IAA and tryptophan in 5-MT resistant wild carrot cell lines.

Further evidence that not all of the intracellular tryptophan is available for the production of IAA was provided by the observations of Phelps and Sequera (1968). These authors showed that significantly higher levels of indole metabolites were recovered from the use of tryptamine as a precursor for IAA biosynthesis than from tryptophan in tobacco bud tissue indicating that the tryptophan was being metabolised in alternative intracellular pathways or was not available at the site of IAA biosynthesis.

Rekoslavskaya et al (1986) reported that up to 80% of cellular tryptophan was not available for cytosolic IAA biosynthesis and Butenko et al (1979) observed that ^{14}C tryptophan was very actively incorporated into the methanol-insoluble fractions of Dioscorea deltoidea tissues. This suggested that a significant amount of the amino acid was being metabolised in protein synthesis.
The regulation of tryptophan availability in plant cells has recently been considered by Law and Hamilton (1987). These authors have suggested a mechanism by which plant tissues could distinguish between the tryptophan available for IAA biosynthesis and that required in other anabolic processes. The authors have proposed that the D-isomer of

tryptophan is the active form for IAA biosynthesis in the tryptophan pool and that racemisation from L to D-tryptophan was under the control of GA_3.

An alternative proposition of a mechanism for the regulation of the levels of tryptophan entering IAA biosynthesis is that the enzymes in the biosynthetic pathway are subject to regulation. If this were the case, the most efficient stage of regulation would be the initial conversion of tryptophan to IPyA or tryptamine. El Bahr et al (1987) has suggested that the affinity of L-tryptophan for the enzyme tryptophan aminotransferase may determine the rate of biosynthesis from tryptophan in tobacco callus cultures. Alterations in the activity of the aminotransferase and also tryptophan dehydrogenase (for conversion to tryptophol) did not, however, appear to co-incide with alterations in the levels of either tryptophan or IAA. This would appear to suggest that this factor alone could not be the determining one in IAA biosynthesis.

Some authors have examined feedback regulation of other enzymes involved in IAA biosynthesis. Percival et al(1973) observed an auxin specific inhibition of indole-3-ethanol oxidase which converts tryptophol to IAAld. In this same study, tryptophan decarboxylase, tryptophan amino-transferase and IAAld oxidase were found to be insensitive to auxin. It must be considered here that the enzymes thought to be involved in IAA biosynthesis not totally specific for IAA biosynthesis and feedback inhibition by high levels of auxin would not necessarily

correspond to the limitation of IAA biosynthesis *in vivo*.

The possibility that tryptamine was produced as a major metabolite of ^{14}C tryptamine in the *Lactuca* callus tissues suggested that these cultures were capable of utilising a pathway of IAA biosynthesis not often reported in plant tissues (Schneider and Wightman 1974). Tryptophan decarboxylase, the enzyme which converts tryptophan to tryptamine has been reported in several plant species analysed by Gibson etal (1972 a and b) including tobacco, tomato, barley, wheat and cucumber. Peas, mung beans and pumpkins did not, however appear to contain this enzyme in this same study. Liu etal (1978) was unable to detect tryptophan decarboxylase in *N. glauca*, *N. langsdorfii* or in the hybrid tissue of both, discounting the proposal that the tryptamine pathway was involved in the induction of auxin-independent growth in these tissues. Phelps and Sequera (1968) also identified ^{14}C tryptamine as a metabolite of ^{14}C tryptophan in terminal bud tissue of tobacco and more recently, Rausch et al (1985) also observed tryptamine as a metabolite of radiolabelled tryptophan in potato crown gall cultures.

The inability to detect more indole metabolites of ^{14}C tryptophan in this present work may have been partially due to the labile nature of the intermediates thought to be involved in IAA biosynthesis including as IPyA, IAA and IAAld. The inherent lability of indoles presents problems in the accurate determination of the *in vivo* IAA metabolism due to the ease of tryptophan to convert to IAA by chemical

means (Epstein etal 1980). For this reason, adequate precautions must be taken to minimise such chemical conversions and maximise the stability of indoles present. The use of anti-oxidant substances such as sodium diethyldithiocarbamic acid have been shown to stabilise indole compounds in plant extracts (Ernsten et al 1986) and redistilled solvents are essential.

Bandurski et al (1977) has proposed that rather than endogenous IAA levels being determined by de novo biosynthesis from tryptophan, the formation and subsequent hydrolysis of IAA conjugates may be responsible for the homeostatic regulation of IAA levels in plant tissues. Evidence for this hypothesis was provided by a measurable alteration in the balance of free:bound IAA levels in Zea mays seedlings in response to a growth-inhibitory light flash. A significant reduction in IAA levels was measured in the auxin-dependent Lactuca callus tissue in response to culture in the dark which also caused a reduction in growth rate over a 6 week period. The mechanism by which these levels of free IAA were reduced in the dark is not clear but could have been the result of an increased catabolism of IAA, a decrease in auxin protector levels, an increase in the level of conjugate forms of the auxin or perhaps an increased efflux of IAA into the surrounding growth medium. The estimates of total IAA for the non-habituated tissue did not demonstrate any significant difference between these levels in light and dark grown tissue. This was in part due to the problems of obtaining a pure enough extract

for accurate quantitative analysis as the variation between replicates was very high. The extent of contamination of the IAA recovered from the hydrolysed extracts was not established and therefore the estimations may only be considered as preliminary. No additional purification steps were introduced for the analysis of the hydrolysed extracts.

Similarly, Bandurski and Schulze (1974) did not purify hydrolysed extracts of Zea mays tissues any more thoroughly than non-hydrolysed extracts from the same source but the necessity for further purification is dependent on the tissue type and these authors employed extensive purification steps (DEAE, cellulose, Sephadex LH20, TLC and GC-MS prior to IAA estimation by U.V. detection and detection using Ehrman reagent (based on a mixture of Salkowski and Ehrlich's reagent) in purifying the Zea mays seedling tissue. Iino and Carr (1982) also purified both hydrolysed and non-hydrolysed extracts by the same procedure but more thoroughly than in this present work using PVP and polyamide TLC and finally quantified IAA levels using the 2-MIP assay followed by a GC-MS validation. This present work may, therefore, only be regarded as a preliminary attempt to quantify the levels of esterified IAA in Lactuca callus tissue and further purification would certainly be required to reduce sample weight sufficiently for the extracts to be analysed by the method developed for the analysis of free IAA levels in this tissue.

High levels of conjugates have been measured in plant tissues. Cohen and Bandurski (1982) have demonstrated that as much as 98% of IAA may be conjugated in plant tissues. Ninety five percent of the IAA measured in dehusked Avena kernels by Percival and Bandurski (1976) was found to be bound up in conjugate forms. Quantitative estimates of IAA conjugates in tissues other than cereal seeds and seedlings remains limited, although Bandurski and Schulz (1977) did examine levels of both ester and amide conjugates in a variety of plant species. Amide conjugates were released by a more rigorous hydrolysis than ester forms (ie. 100°C for 3 hours in 7N NaOH under a stream of N_2 gas)(Bandurski and Schulz 1977).

Some species specificity was observed by Bandurski and Schulz (1977) with regard to the nature of conjugates produced. Esters were found to be the predominant form of conjugate in cereals, while amides were more abundant in legumes.

Most of the work concerning conjugates of IAA has been in obtained from work on cereal seeds and developing tissues, although Mousedale (1982) did measure free and esterified forms of IAA in germinating seeds of Prunus domestica and found that 80% of the IAA was in ester forms prior to endocarp formation.

The nature and extent of conjugation in tissue cultures is less well studied. IAA-amino acid conjugates of have, however, been employed as slow release forms of auxin (Hangarter et al 1980, Caruso 1987) demonstrating that

tissues are capable of hydrolysing these conjugates *in vivo*. That ^{14}C IAA can be converted to amino acid conjugates by tobacco crown gall cultures (Rausch et al 1986, Vijayaraghavan and Pengelly 1986) demonstrates that tissue cultures are also capable of forming these auxin derivatives. It is therefore feasible that the formation and hydrolysis of IAA conjugates may act as a mechanism for the homeostatic regulation of IAA levels in plant tissues. Rausch et al (1986) has proposed that the rapid conjugation of IAA to IAA-Asp in crown gall cultures may be the mechanism by which transformed tissues are able to maintain IAA levels similar to those in non transformed tissues. They measured very high levels of this form of amide conjugate in crown gall tissues.

The levels of IAA-Asp formation in tobacco crown gall cells analysed by Viyahavan and Pengelly (1986) were, however, very low, in contrast to the results of Rausch et al (1986). As much as 45% of the IAA taken up by *Parthenocissus tricuspidata* callus cultures was conjugated to several different amino acids. It is interesting that the only conjugates of IAA found in auxin-independent tissue cultures in these ^{14}C IAA feeding experiments in this work were amide forms. Viyahavan and Pengelly (1986) detected very little esterified auxin in both tobacco pith and crown gall cultures, but did observe that ^{14}C NAA was rapidly converted to esterified forms.

It may not be only quantitative alterations in the levels of free and bound IAA which are important but also

the nature of conjugates produced. There seems to be some specificity with regard to the ability of different AA conjugates of IAA to withstand peroxidase attack. The rate of hydrolysis and subsequent biological activity of amino acid conjugates appears to be related to the nature of the adjoined amino acid moiety and there is evidence to suggest that IAA does not always bind to the most abundant amino acid available (Hangarter et al 1980). This would suggest that the nature and the extent of conjugation may both play a role in the regulation of IAA levels in plant tissues.

This work suggests that further investigation of the metabolism of radiolabelled auxin in the habituated and non-habituated Lactuca tissues would be worthwhile to determine the nature (ester or amide) and extent of IAA conjugation in these tissues and this would then enable the development of a more relevant purification technique for the quantification of IAA conjugates in these tissues. This would involve further purification of the hydrolysed extracts.

In attempting to determine any possible regulatory function of IAA conjugates it is necessary to extend the knowledge of these conjugate forms of IAA to species and developmental stages other than the well characterised cereal seed development system. Tissue culture could provide an ideal system for identification and measurement of alterations in free and bound IAA levels in response to alterations in environmental conditions within the in vitro system, such as growth-inhibitory light regimes.

Dedifferentiated tissues provide an excellent system for this type of study because of the ease with which growth rates may be determined and the relative uniformity lends itself to metabolic analysis.

Chapter 5

The effect of auxin antagonists and analogues on the uptake and incorporation into DNA of ^{3}(H) thymidine in habituated tissues

5.1 Introduction

Compounds antagonistic to the binding of auxin to auxin-binding sites have been demonstrated in coleoptile tissue of maize and Curcurbita (Jacobs and Hertel 1978, Ray et al 1977). Rausch et al (1984) employed auxin antagonists to displace endogenous IAA from auxin binding sites in a tobacco crown gall culture in an attempt to determine the effect of endogenous IAA on the uptake of sugars and amino acids by this tissue, by eliminating auxin activity. Auxin antagonists demonstrate little, if any auxin activity in auxin bioassays (Katekar 1979) and are therefore useful in the study of endogenous auxin activity as they displace bound auxin from binding sites, yet elicit no auxin response.

Auxin antagonists are often required at high concentrations in order to displace bound auxin from binding sites in plant tissues. Murphy et al (1980) observed that the antagonist 2-NAA only began to compete with bound auxin in maize membrane preparations at concentrations of 10μM and antagonists at concentrations of 10μM and above were required by Ray et al (1977) to cause a significant reduction in auxin binding in maize coleoptile tissue.

The aim of this following work was to employ auxin antagonists to displace endogenous IAA from binding sites in habituated Lactuca tissue in an attempt to determine the role of endogenous auxin in the regulation of proliferation in this tissue. ^{3}(H) thymidine uptake and incorporation into DNA were monitored in the habituated tissue in the presence of auxin antagonists in an attempt to determine the role of endogenous auxin in the uptake of essential precursors for growth and in DNA synthesis.

5.2 Materials and methods

Habituated Lactuca callus tissue was employed in these experiments. This tissue was isolated by Savigear (1985) and was maintained on Murashige and Skoog medium (Murashige and Skoog 1962) without additional growth regulators.

Chemicals

The auxins, indole-3-acetic acid and 1-naphthylacetic acid, antagonist, 2-naphthalacetic acid and analogues, benzoic acid and phenoxyacetic acid were all purchased from Sigma Chemical Co.. The antagonists 1-naphthoxyacetic acid and chlofibric acid were obtained from Aldrich. Methyl benzethonium hydroxide was also supplied by Sigma Chemical Co. 6-^{3}(H)-thymidine (185 GBq/m mol) was supplied by Amersham International plc. and Optiphase "Safe" scintillant was obtained from LKB, Fisons plc.

Preparation of callus tissue for ^{3}(H) thymidine feeding experiments

A pre-incubation period in liqiud medium was found useful as an aid to the dispersion of callus tissue in test dishes, which provided a more uniform uptake of the ^{3}(H) thymidine during short time-course experiments. 100mg of callus tissue was weighed aseptically, in approximately 20mg explants, into 3 x 1 cm plastic petri dishes (Sterilin Ltd.) using a sterile spatula. 1ml of Murashige and Skoog liquid medium (Murashige and Skoog 1962, pH 5.5, 2 % sucrose) was then added to the tissue under sterile conditions using a Gilson automatic pipette (Gilson, Anachem, Luton, Beds.) and the dishes were then sealed with a thin strip of parafilm (American Can Co. Greenwich CT06830). The cultures were incubated at 25°C on a rotary platform (60 rpm) in continuous light of 685 μ mols m s^{-1} for 24 hours.

Feeding ^{3}(H) thymidine to callus tissue

After the 24 pre-incubation period, the dishes were re-opened in a laminar airflow cabinet and both the radiolabelled thymidine and test compounds (either auxin, antagonist or analogue) were added using a Gilson pipette. Test compounds in 5mM MES buffer (pH 5.5) were added in 0.5ml volumes at a range of concentrations from 1-400 μM. Control dishes received 0.5ml of pure buffer. The ^{3}(H) thymidine was then added to all cultures (74 KBq/ 0.5ml) and the dishes were resealed with parafilm. The cultures

were incubated for set time periods (from 0 to 160 minutes) before the uptake of the thymidine was halted by the addition of 0.5ml of ice-cold thymidine (5 mM).

At the end of each incubation period, approximately 0.1ml of the incubation medium was extracted by pasteur pipette for pH measurement. This was determined using a pH microelectrode (Russel pH Ltd. Auchtermuchty, Fife). A loopful of medium was also removed and streaked onto Nutrient agar/ Malt extract agar plates (Oxoid Ltd.) for the assessment of bacterial or fungal contamination. Dishes were resealed with parafilm and stored at 4°C prior to incorporation or uptake measurement.

Measurement of ^{3}(H) thymidine incorporation into DNA

DNA synthesis in the Lactuca cells was estimated by the measuremt of ^{3}(H) thymidine incorporation into trichloroacetic acid insoluble material by the method of Ferrari and Widholm (1973).

The contents of each dish were transferred to a 15ml glass centrifuge tube assisted by a small spatula and a glass funnel. The dishes were rinsed with distilled water and this was added to the centrifuge tube. Distilled water was then added to the tube to give a final volume of 10ml. The contents were then centrifuged at 400g for 10 minutes to pellet out the plant material.

The supernatant was carefully removed by pasteur pipette and discarded. Approximately 5 drops of trichloroacetic acid were added to each tube and the tissue

was then homogenised. Distilled water was added to the homogenate to aid transfer to a calibrated glass tube in which the volume was made upto 7.5ml. The contents of the calibrated tube were thoroughly mixed using a whirlmixer and then samples of 1.5ml (corresponding to approx. 20mg fresh weight of tissue) were removed and transferred to GFA filter discs (Whatman, 2.5cm) under suction.

The material on the filters was washed with 6ml of a methanol: chloroform: water mixture (12: 5: 3 by volume) containing unlabelled thymidine (5 mM) to dilute out the unincorporated radiolabelled isotope (Ferrari and Widholm 1973). Figure 5.1 demonstrates that 6ml of this mixture was sufficient for the removal of unincorporated radioactivity from this tissue. The counts per minute recovered on filters containing 20mg of tissue were seen to have fallen to a constant level of radioactivity when 6ml of the washing solution was applied.

The washed filters containing the plant samples were dried in a vacuum oven at 100°C for 10 minutes and then carefully transferred to scintillation vials using a pair of forceps. 5ml of Optiphase "Safe" scintillant was added to each vial and the radioactivity determined using a (Wallac) Rackbeta 1217 liquid scintillation counter.

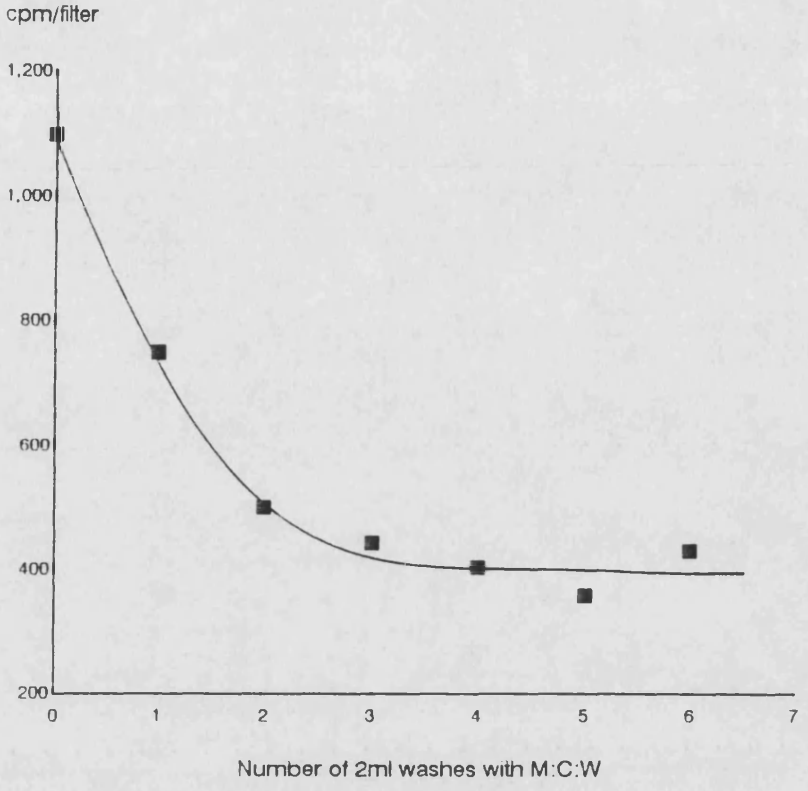


Figure 5.1: Effect of subsequent methanol:chloroform:water (M:C:W) washes on the radioactivity of Lactuca cells.

Measurement of ^{3}(H) thymidine uptake

The uptake of ^{3}(H) thymidine into Lactuca tissues was measured using a method based on that for the determination of glucose uptake into tobacco crown gall cultures (Rausch et al 1984). Sample dishes were removed from the cold and the contents transferred to Whatmann hardened ashless (No. 542) filter discs under suction. The dishes were rinsed with approximately 2ml of distilled water. The tissues on the filter were then washed with 15ml of ice-cold thymidine (5 mM) to dilute out the radiolabelled isotope.

The washed tissue was then transferred from the filter to a 15ml glass centrifuge tube using a small spatula, and 1ml of methylbenzethonium hydroxide was added to each tube to solubilise the tissue. The tubes were then covered with a layer of aluminium foil and placed in a heating block at 60°C for 1 hour. After this incubation, the contents were homogenised to fine particles and 1ml of methanol was added to give a final volume of 2ml in each tube. Samples of the homogenate (0.5ml, approx. equivalent to 25mg fresh weight of tissue) were transferred to scintillation vials and 4.5ml of scintillant (Optiphase "Safe") was added. The radioactivity was then determined as above.

5.3 Experimental

An initial experiment was carried out to determine the most effective auxin antagonist for the displacement of endogenous auxin from auxin-binding sites in the habituated tissue. DNA synthesis, determined by ^{3}(H) thymidine incorporation into the DNA of the Lactuca cells, was measured in the presence of one auxin, IAA, and three antagonists; PCIB, 2-NAA and 1-NOA. Control cultures received pure buffer in place of the test compounds. A test concentration of 100μM was chosen to ensure displacement of bound auxin. DNA synthesis measured in the cells in the presence of the test compounds was calculated as a percentage of control DNA synthesis and the results are presented in Figure 5.3. No attempt was made to monitor callus growth by fresh or dry weight.

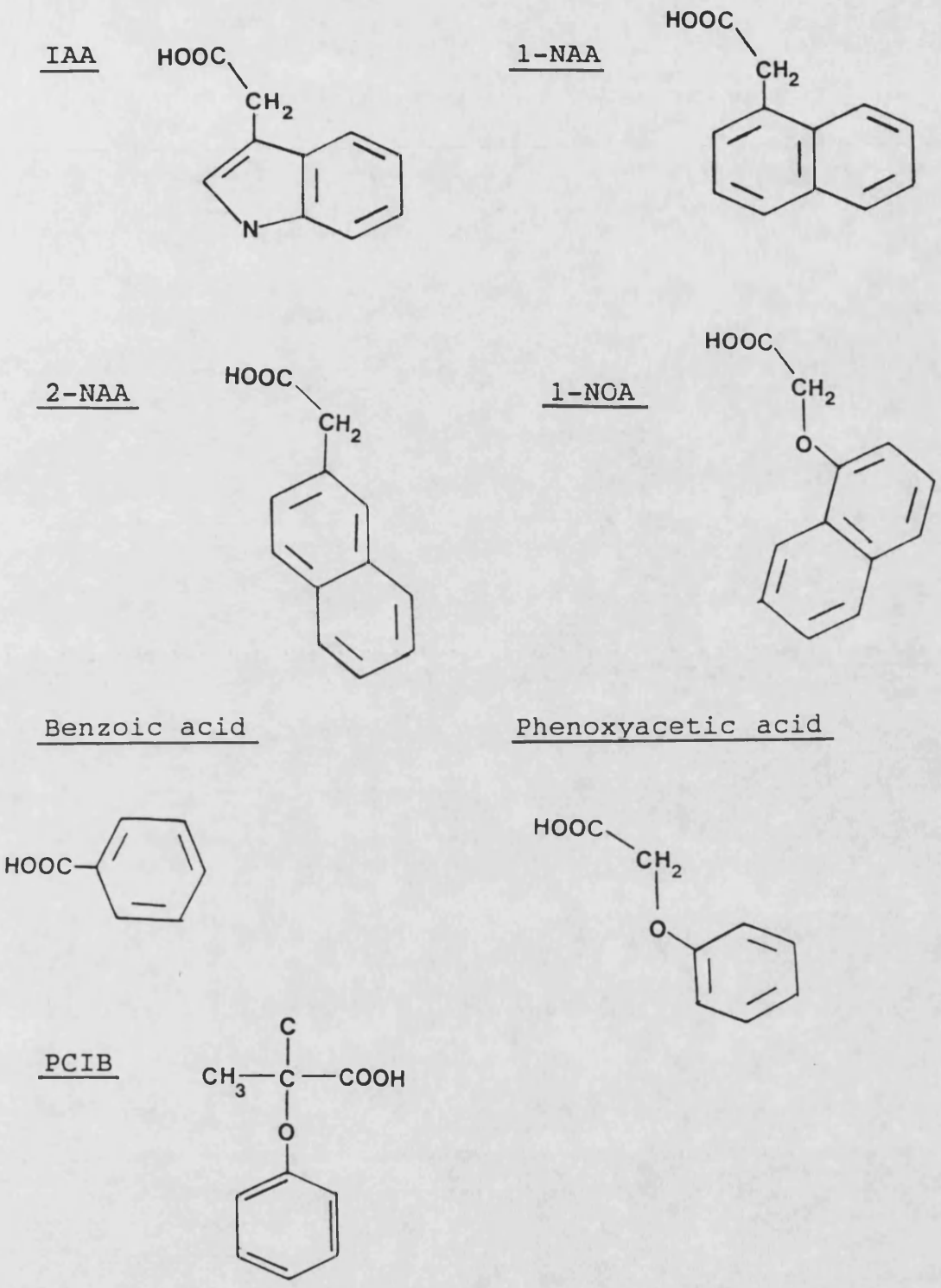


Figure 5.2 : Chemical structure of the auxins, antagonists and analogues used in this work.

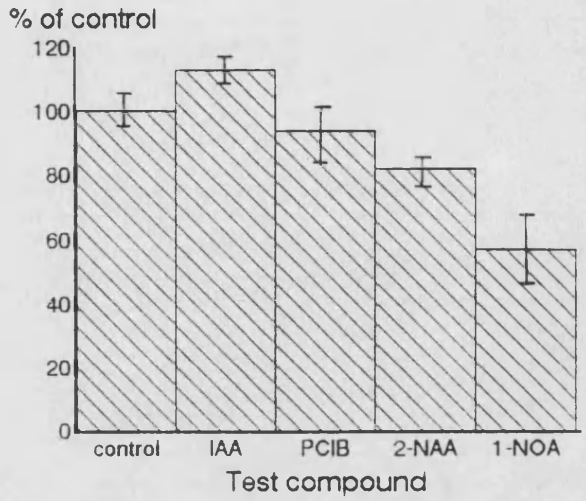


Figure 5.3 : Effect of the addition of IAA and three auxin antagonists on DNA synthesis in Lactuca callus tissue. Data are presented as a percentage of control DNA synthesis. Error bars represent standard error of the mean.

Figure 5.3 demonstrates that 1-NOA was the most effective compound tested at the chosen concentration of 100 μM for the inhibition of DNA synthesis in habituated Lactuca callus tissue. Inhibition of this DNA synthesis by 1-NOA was thought to be via the displacement of endogenous bound auxin in the tissue. No other compound caused a significant reduction in DNA synthesis at this concentration. The antagonist, 1-NOA was therefore chosen for use in preliminary investigations of the time course of antagonist mediated inhibition of DNA synthesis.

Habituated cultures were exposed to 100μM 1-NOA for

varying periods of time from 0 to 160 minutes and both the incorporation of ^{3}(H) thymidine into DNA and its uptake into the tissue were followed in separate experiments. The rates of DNA synthesis and thymidine uptake measured in the presence of 1-NOA were compared to rates measured in control cultures.

The rate of ^{3}(H) thymidine uptake into the tissues and its incorporation into DNA were both found to be constant in the absence of the auxin antagonist (Figure 5.4). The incorporation of the nucleoside into DNA demonstrated a lag period of up to 20 minutes before a constant reduced rate (approximately 75% of control) of DNA synthesis was achieved.

The effect of 1-NOA on the uptake of ^{3}(H) thymidine was followed in an attempt to determine if the observed reduction in DNA synthesis was caused by a reduction of thymidine uptake at the plasmalemma. The apparent reduction in the rate of thymidine uptake into the tissues on the addition of 1-NOA (Figure 5.5) suggested that inhibition of uptake at the plasmalemma was, at least, partially responsible for the observed reduction of DNA synthesis caused by the auxin antagonist or the reduction in DNA synthesis was reducing the rate of uptake of the precursor.

An attempt was made to reverse the inhibition of the antagonist, 1-NOA on thymidine uptake by adding both 1-NOA and the auxin 1-NAA simultaneously to the cultures. Figure 5.6 shows that in contrast to any reversal, inhibition of

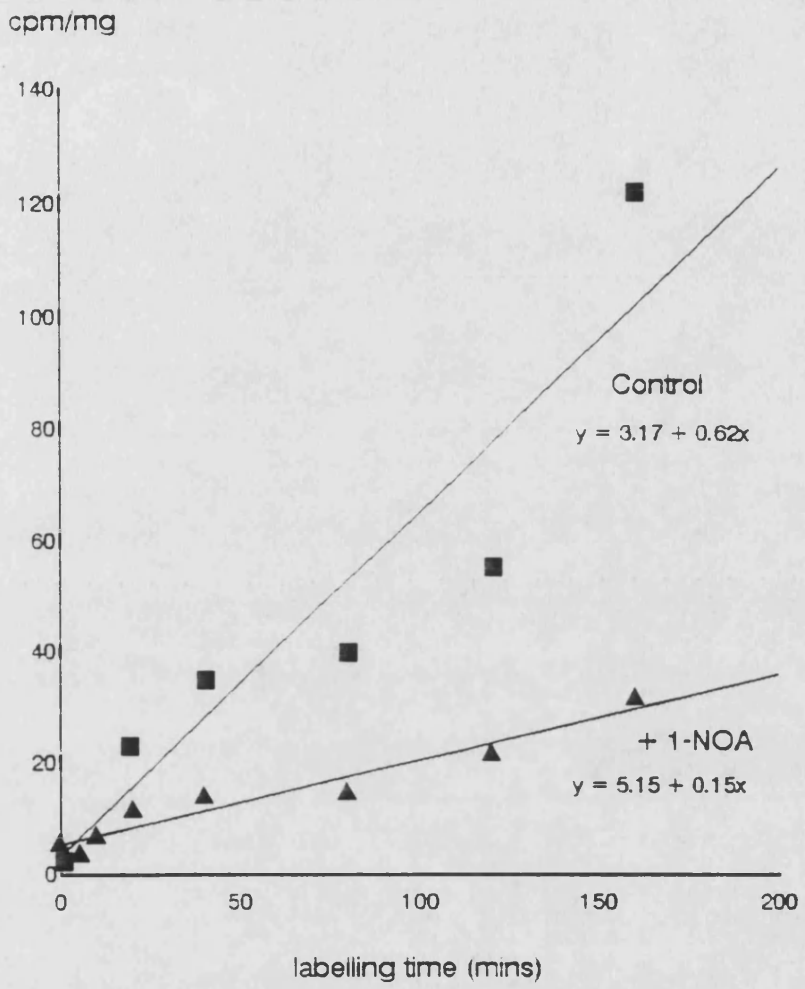


Figure 5.4 : Time-course of effect of 1-NOA on DNA synthesis.

For statistics see Appendix.

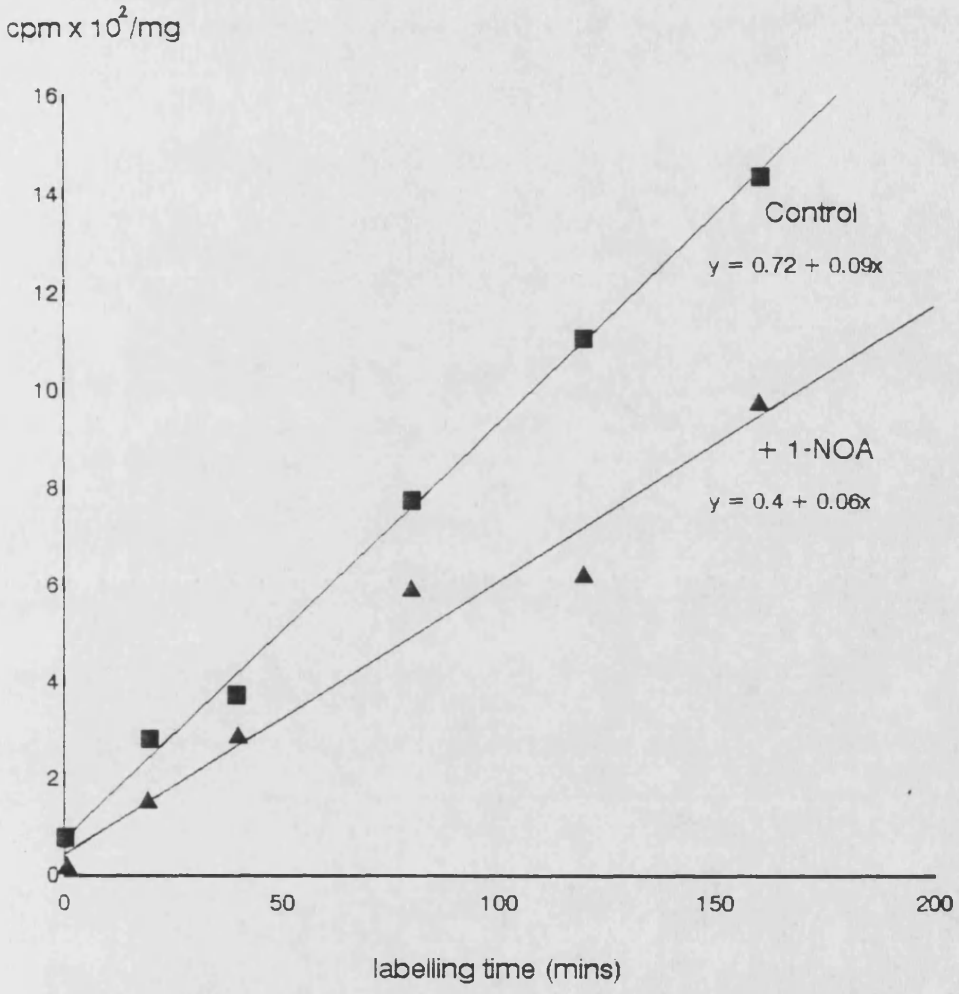


Figure 5.5 : Time-course of effect of 1-NOA on ^{3}(H) thymidine uptake.

For statistics see Appendix.

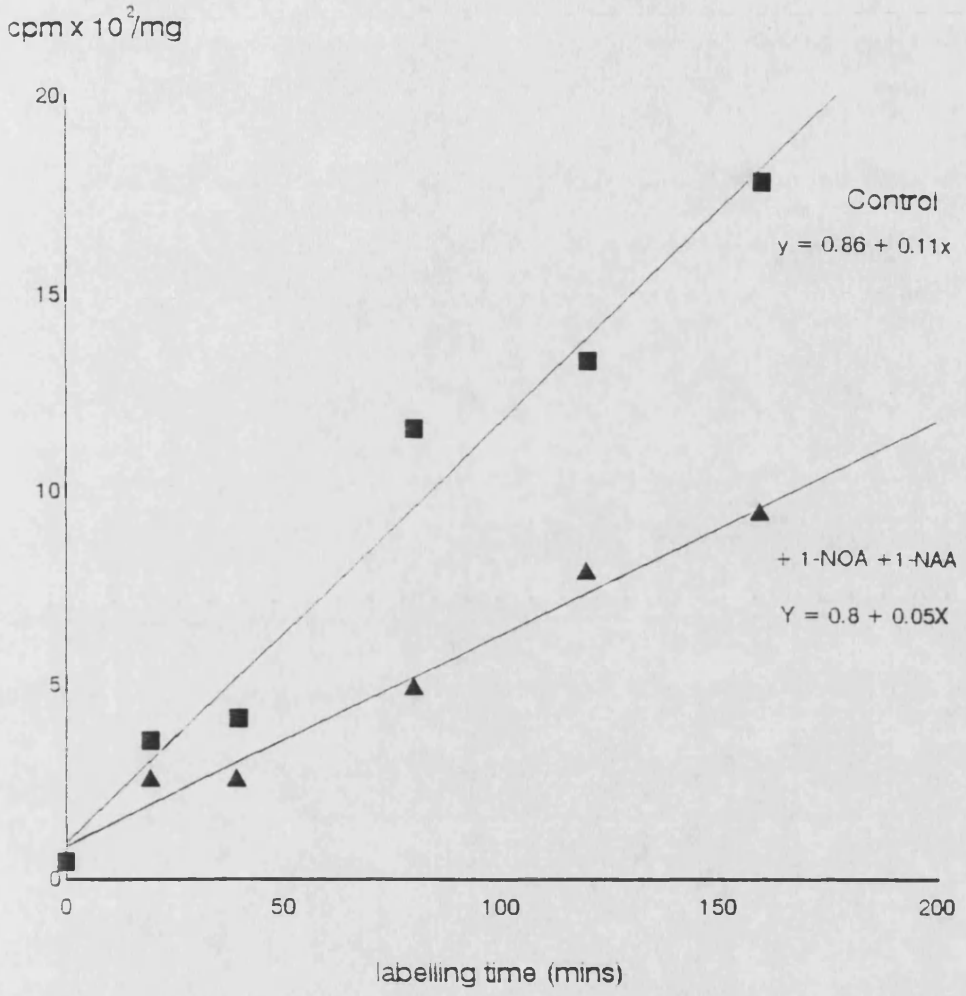


Figure 5.6 : Time-course of effect of 1-NAA and 1-NOA on ^{3}(H) thymidine uptake.

For statistics see Appendix.

thymidine uptake had been increased (34-55%). 1-NAA was, however added at 100μM, which, although suitable for the displacement of the an auxin or antagonist from the binding site, was a growth-inhibitory concentration of auxin. The habituated tissues are self sufficient in auxin production and the addition of excess auxin could quite significantly inhibit cell growth involving the requirement for solute uptake.

To determine whether other reported auxin antagonists could elicit a similar inhibitory response on thymidine uptake, other auxins (IAA and 1-NAA) and antagonists (2-NAA) were tested at a range of concentrations (25 to 400μM). Figures 5.7 to 5.10 illustrate the response of the habituated tissue to the addition of these compounds. Both auxins and antagonists were seen to inhibit the uptake of thymidine in the habituated tissue. 1-NAA was found to be one of the most efficient inhibitors of thymidine uptake at the concentrations applied (Figure 5.9).

In order to determine the specificity of the action of the antagonists on thymidine uptake inhibition, inactive auxin analogues were also tested. The effect of these compounds on thymidine uptake was followed over the same concentration range. Figures 5.11 and 5.12 clearly demonstrate that the analogues exhibited a different effect on thymidine uptake to the antagonists which suggested that the effects of the auxin antagonists was specific to their ability to displace bound auxin in the tissue and not the result of a general poisoning effect on the tissue.

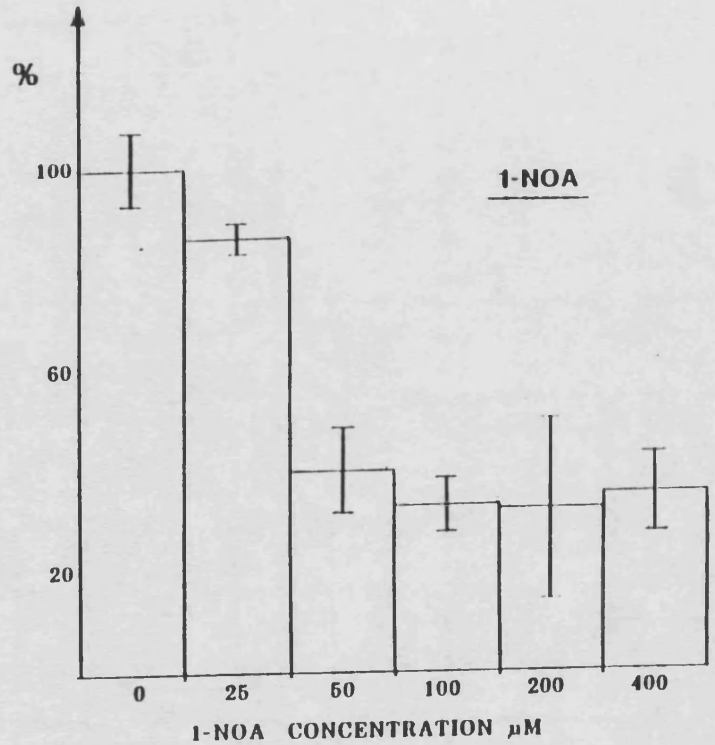


Figure 5.7

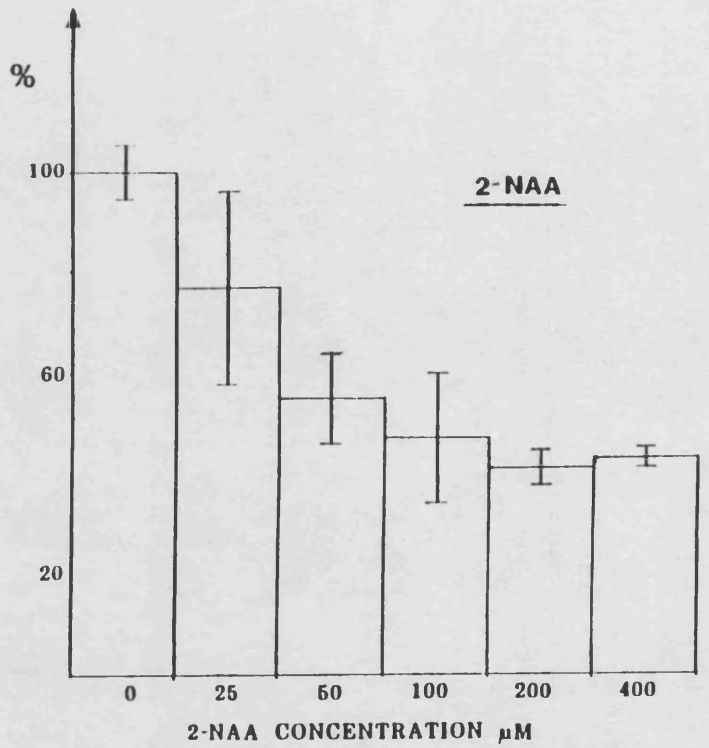


Figure 5.8

Figures 5.7 and 5.8 : Effect of antagonist concentration on ^{3}(H) thymidine uptake. Error bars represent % standard error of the mean.

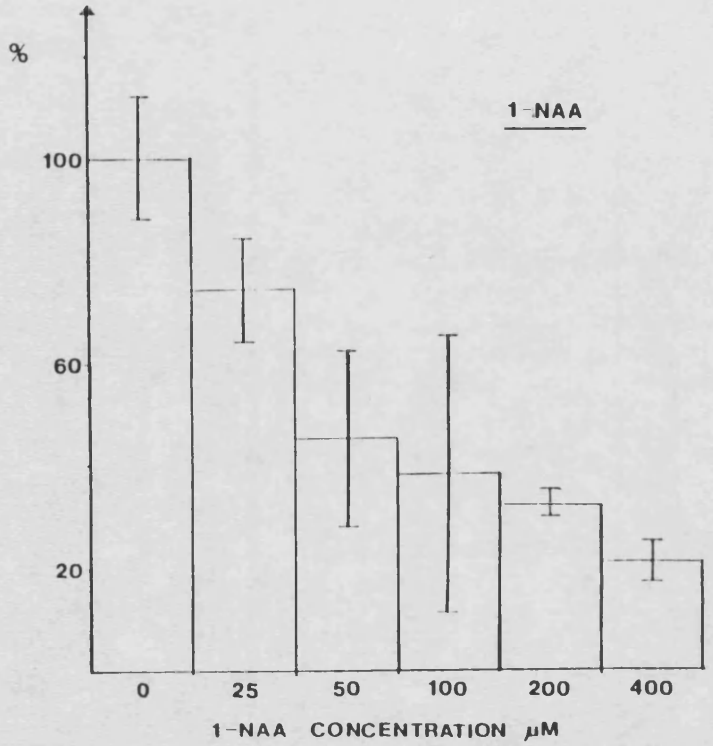


Figure 5.9

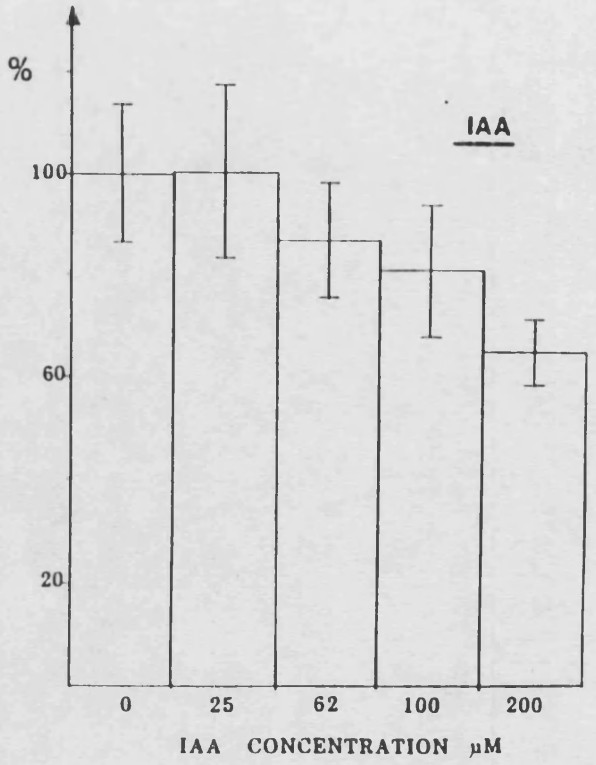


Figure 5.10

Figures 5.9 and 5.10 : Effect of auxin concentration on ^{3}(H) thymidine uptake. Error bars represent % standard error of the mean.

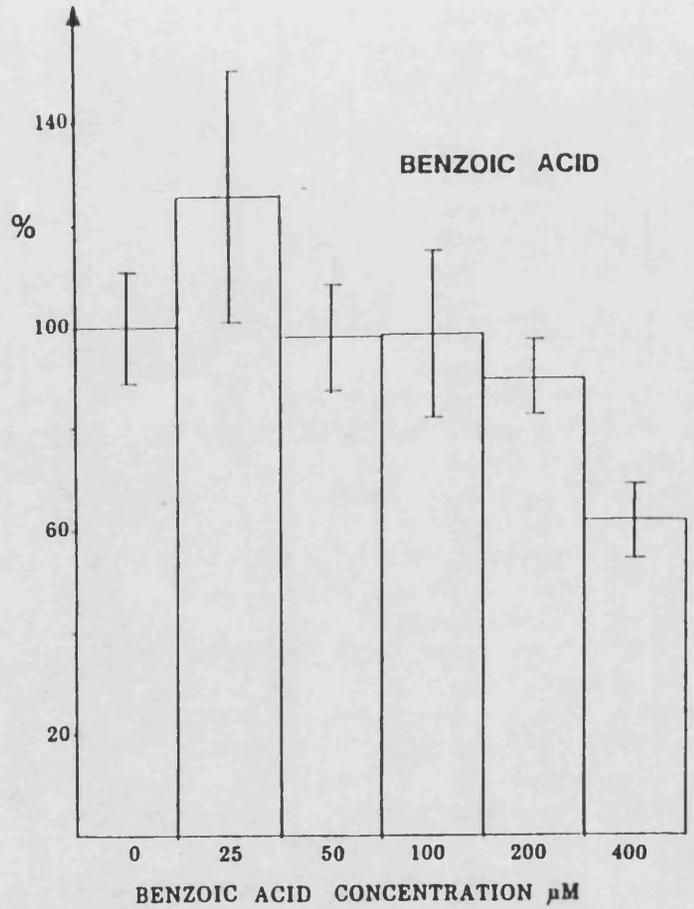


Figure 5.11

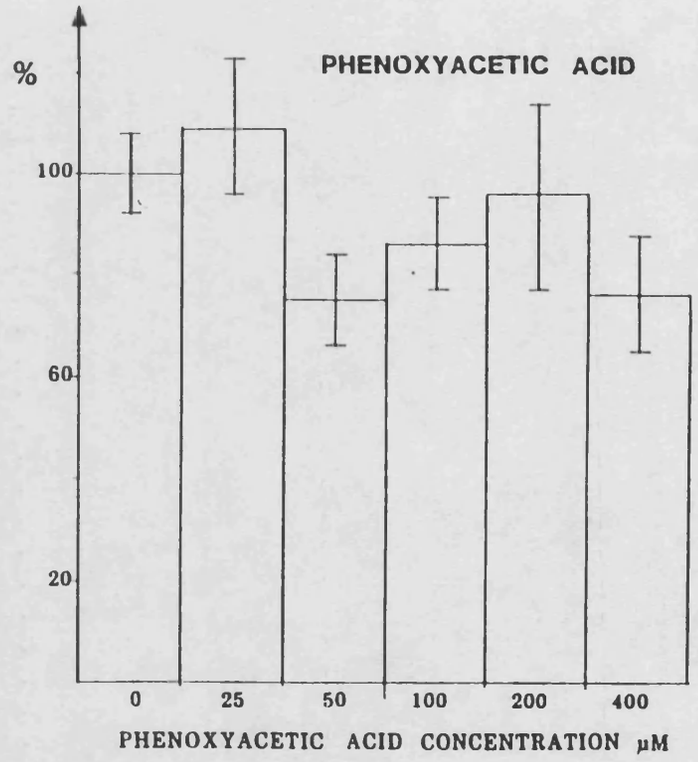


Figure 5.12

Figures 5.11 and 5.12 : Effect of analogue concentration on ^{3}(H) thymidine uptake. Error bars represent % standard error of the mean.

5.4 Discussion

The rate of DNA synthesis and thymidine uptake into habituated Lactuca callus tissue can be reduced by incubation with an auxin-binding antagonist, such as 1-NOA (Figure 5.4). These compounds are able to displace bound auxin from specific auxin-binding sites of both plasmalemma and tonoplast in Cucurbita and maize coleoptiles (Jacobs and Hertel 1978, Dohrmann et al 1978). Although they possess affinity for auxin-binding sites, these compounds,(eg. 1-NOA and 2-NAA) do not appear to possess auxin activity in bioassays such as the split pea stem and coleoptile straight growth assays (Katekar 1979). The presence of an antagonist will, however, interfere with the level of endogenous auxin-binding in plant tissues and will therefore also affect endogenous auxin activity. The reduction of DNA synthesis and thymidine uptake measured in this habituated tissue in the presence of the antagonist, 1-NOA, indicated the involvement of auxin in the regulation of both processes. The nature of this involvement is, however, unclear.

The observed reduction of thymidine uptake at the plasmalemma (Figure 5.5) could be the limiting factor in its incorporation into DNA. In this way, auxin could have a primary role in the regulation of solute uptake into plant cells as has been suggested by Rausch et al (1984). Rausch et al (1984) suggested that such an effect could be elicited through auxin enhancement of the electrochemical gradient across the plasmalemma stimulating the symport of

solutes into the cells.

Alternatively, a reduction in certain auxin-mediated intracellular processes could be responsible for the reduction in the amount of thymidine required and taken up by the cell. Furthermore, there may be direct auxin regulation of DNA synthesis in addition to the apparent regulation at the plasmalemma. There is, however, great difficulty in studying auxin involvement in processes such as DNA synthesis and thymidine uptake in isolation of the other intracellular processes in which auxin is thought to be involved.

^{3}(H) thymidine uptake was not seen to be promoted by IAA at any of the concentrations tested (Figure 5.10). This inability to detect IAA stimulation of ^{3}(H) thymidine uptake in the tissue may be an indication of altered sensitivity in this tissue to applied auxin as a result of auxin-habituation but is more likely to be the result of the use of concentrations of auxin too high for growth promotion in this tissue. Additionally, auxin receptors involved in the regulation of thymidine uptake at the plasmalemma may have very high affinity for auxin and thus saturate at low auxin concentrations. The IAA added to the tissue was excess, perhaps as a result of supraoptimal endogenous IAA levels and exerted a similar, less severe effect on the uptake as the antagonists. It is possible that IAA stimulation may have been detectable at concentrations well below 25μM although this was not demonstrated in this work. Rausch et al (1984) reported the stimulation of glucose uptake in

potato crown gall cultures by 100μM IAA. This would suggest that either the mechanisms controlling the uptake of thymidine and glucose are different or that the sensitivity of the two auxin independent tissues to auxin are very different.

The addition of the auxin antagonists 1-NOA and 2-NAA have similar effects on the uptake of glucose (Rausch et al 1984) and the uptake of thymidine (Figures 5.7 and 5.8). The results suggest that auxin involvement is common to both mechanisms. The uptake of organic solutes such as thymidine and glucose might be expected to have a common control mechanism, as both are essential precursors for growth.

Further studies by Rausch et al (1984) on amino acid uptake in potato crown gall cultures showed that the effect of adding auxin antagonists on the uptake of amino acids and glucose were not the same even in the same tissue. This suggests that auxin is involved in regulating the uptake of various essential precursors into plant cells but not necessarily in the same manner.

The effect of increasing the concentration of antagonists 1-NOA and 2-NAA applied on thymidine uptake (Figures 5.7 and 5.8) might indicate something of the nature of thymidine transport into these cells. There appear to be at least two separate modes of thymidine uptake in this tissue demonstrated in Figures 5.7 and 5.8). One mode appears to be auxin-sensitive and appears to be saturated at antagonist concentrations of 50μM and above.

The second mode of uptake appears to be unaffected, even by the addition of excessively high concentrations of antagonist and is therefore apparently auxin-independent.

It must be considered that the effect of auxins and antagonists on the uptake of thymidine could feasibly be the result of a general poisoning effect on the tissues caused by very high test concentrations. The auxin analogues, benzoic acid and phenoxyacetic acid were employed to try and determine the specificity of the effect of the antagonists on thymidine uptake. Figures 5.11 and 5.12 appear to suggest that the effects of 1-NOA and 2-NAA on thymidine uptake were both significant and specific, and not apparently due to a general poisoning of the tissue. The addition of these analogues have no significant effect on thymidine uptake until at very high concentrations, where significant depression of uptake was evident. These analogues do not appear to have high affinity for auxin-binding sites *in vitro* (Dohrmann et al 1978) and possess slight, if any, auxin activity (Katekar 1979).

The reason why 1-NAA, which possesses strong auxin activity should behave more like 1-NOA and 2-NAA than auxin, IAA in its strong "antagonistic" effect on thymidine uptake is not clear (Figure 5.9). Rausch et al (1984) found that the auxins 1-NAA and 2-NOA were inhibitory to glucose uptake in crown gall tissues of potato, even at concentrations of 25μM, whereas stimulation of this uptake was observed at 100 μM IAA. It was suggested by the authors that supraoptimum levels of auxin may occur in auxin-

independent tissues at lower levels of a strong auxin such as 1-NAA, than with IAA. It is interesting to note that 1-NAA is often reportedto have a higher affinity for auxin-binding sites *in vitro* than IAA (Dohrmann et al 1978).

There is difficulty in the interpretation of these results regarding the effect of auxin on the regulation of thymidine uptake and incorporation into DNA due to the lack of information on thymidine uptake into plant cells. Thymidine transport into cultured cells has, however, been characterised in certain mammalian cells such as Novikoff rat hepatoma, chinese hamster ovary and mouse (fibroblast) L cells (Wohlheuter et al 1979). This was made possible through a combination of rapid mixing/sampling techniques with integrated rate analysis. The kinetic behaviour of the nucleoside transporter was found to be different from that of the incorporation system. The free nucleoside equilibrates rapidly, within seconds, but the long-term rate limiting factor in the incorporation was the slower rate of phosphorylation of the intracellular nucleoside. Although inadvisable to lean heavily on animal systems for information, it is interesting to consider that the regulation of thymidine uptake in plant cells could be similar, with auxin perhaps involved at the stage of intracellular phosphorylation of the nucleoside as opposed to direct regulation of uptake at the plasmalemma.

General Discussion

Since Gautheret first identified habituation for auxin in the 1940's, several authors have studied the phenomenon of habituation for plant growth regulators. The mechanisms by which the requirements for auxin and cytokinin of *in vitro* grown plant tissues are altered during culture is not known, but the spontaneous occurrence of habituation in tissue culture demonstrates that certain factors of the *in vitro* environment are responsible for stable alterations in the phenotype of cultured plant cells.

One obvious component of the tissue culture environment which may effect the genome modifications necessary for such phenotype alterations are the growth regulators supplied to cultured plant cells in the culture medium. The exposure of cultured plant tissues to exogenous growth regulators has been shown to affect the stability of the plant cell genome (Bayliss 1973, Orton 1980) and to induce alterations in the genetic material such as the production of new satellite DNA (Grisvard and Tuffer 1980) and the preferential replication of specific DNA sequences (Nagl and Rucker 1972, Kessler 1973).

That growth regulators have some influence on the induction of habituation has also been suggested by the results of several authors. Both Gautheret (1957) and Buiatti (1971) have reported that variation in the concentration of auxin supplied to tissue cultures has a considerable influence on the percentage of tissues able to habituate and short-term auxin pretreatments have been

shown to induce the capacity of certain tissue cultures to grow in the absence of additional auxin (Syono and Furuya 1974).

In this present work, callus tissues initiated from the leaf discs of Lactuca sativa var.L were observed to habituate readily. Habituation was demonstrated by the ability of tissues to grow on basal medium for a period of up to 9-12 weeks following transfer from a medium containing auxin and cytokinin. Habituated tissues were isolated from the Lactuca cultures after only short auxin and cytokinin treatments, with a maximum period of 28 days exposure to these growth regulators. Callus cultures initiated and grown for 28 days on a medium containing NAA and kinetin gave rise to a higher proportion of habituated tissues than cultures initiated on a medium with auxin treatments of only 1, 2 or 4 days. This may have been the result of greater vigour of growth in the tissues given the longer auxin treatment. In cultures grown for extended periods of up to 42 weeks on a medium containing NAA and kinetin, no auxin-habituated tissues were isolated after transfer to a medium without growth regulators (Figure 3), presumably as a result of cell selection for auxin and cytokinin requirement in the tissue over time in culture on a medium containing these growth regulators.

The habituated tissues of Lactuca isolated in this work arose as patches of growth on the surface of auxin-dependent tissues (Plate 6). Thus, a specific balance of growth regulators may be required to induce habituation,

in this case supplied by the underlying tissue. These freshly isolated habituated tissues were not used in biochemical studies in this project as an older line, isolated by Savigear (1985), was found to be a more uniform and bettter characterised tissue and therefore preferable for use in this type of work.

NAA was found to be an absolute requirement for the induction of callus from Lactuca leaf tissues in this work but the involvement of auxin in the induction of habituation is far more difficult to demonstrate as habituation occurs spontaneously and some species and genotypes appear to habituate quite readily. For such a demonstration, the tissue cultures used would have to be demonstrated as auxin and/or cytokinin-requiring before the influence of growth regulators on the induction of habituation could be examined.

The biochemical mechanisms responsible for alterations in the metabolism of growth regulators in autonomous plant cells is not known. Intensive research of the crown gall condition, in which autonomy for growth regulators is conferred on plant cells by the integration of bacterial genes into the host cell genome, has shown that part of the induction mechanism of autonomous growth in crown gall lies in the integration of genes which code for enzymes involved in the biosynthesis of IAA and zeatin (Bevan and Chilton 1982). In habituated tissues no transformation with exogenous genetic information takes place but other genetic modifications may occur under the

influence of the *in vitro* environment which could result in the expression of auxin and cytokinin-autonomous growth.

Gautheret (1955) and Meins et al (1980) have proposed that habituation results from stable, yet reversible, alterations in the cellular phenotype of plant cells. There is some evidence that quantitative alterations in the genetic component do take place in habituated cells (Durante et al 1986, Cecchini et al 1983). Meins and Lutz (1980) have supported this hypothesis of an epigenetic basis for habituation with statistical evidence which suggested that the rate of conversion of tobacco Havana 425 tissues to the cytokinin-habituated phenotype occurred too rapidly to be the result of a classical mutation. Meins and Binns (1977) have also demonstrated that reversible shifts between a range of habituated states can occur. Meins et al (1980) have suggested that the epigenetic mechanism involved in habituation may take the form of a positive feedback mechanism whereby growth factors either induce their own synthesis or inhibit their own degradation.

Meins (1987) has since suggested that cytokinin-requirement is a genetic trait regulated at two genetic loci, *Hl-1* and *Hl-2* and that mutation of the *Hl-1* locus, results in the expression of cytokinin-autotrophy in a similar fashion to the induction of cytokinin-autotrophy by the locus on the Ti plasmid which codes for isopentenyl-transferase. The possibility that certain forms of habituation expressed in plant tissues result from permanent alterations to the genetic component of cultured

plant cells cannot be disregarded. Several authors have suggested that auxin-habituation may arise from a mutation in genes involved in the regulatory system of growth regulator metabolism in plant tissues (White 1951, De Ropp 1951 and Kandler 1952) and, more recently, Everett et al (1981) have proposed that habituation may arise from either permanent genetic change or stable epigenetic alterations. An Acer cell suspension studied by Everett et al (1981) was thought to have arisen as a result of a mutation, based on the frequency with which the condition arose. This idea that both genetic and epigenetic alterations may be responsible for the induction of habituation could account for some of the anomalies which exist regarding the conditions required to induce habituation in different tissue cultures. No evidence was obtained in this present project to suggest whether habituation in the Lactuca tissues studied in this work arose by a genetic or epigenetic mechanism.

The preceeding discussion demonstrates that no clear evidence exists, at present, to demonstrate the involvement of auxin in the induction phase of habituation. The role of auxin in maintaining auxin-autonomous growth in cultured plant cells has, however, received more attention. The knowledge that the Ti plasmid of Agrobacterium tumefaciens contains genes which are directly involved in the biosynthesis of IAA and zeatin might appear to support the assumption that the expression of these genes in the plant cell results in the overproduction of these growth

regulators, thereby allowing the tissues to grow in the absence of exogenous auxin and cytokinin. There is, however, limited evidence to support this idea.

Original reports, prior to knowledge of the Ti plasmid, indicated that crown galls contained higher levels of IAA than the tissues from which these cultures were isolated (Kulescha and Gautheret 1948). More recently, there have been similar reports of higher levels of IAA in crown gall cultures than in comparative control cultures (Tandon and Arya 1980, Mousedale 1982). However, Pengelly et al (1986) did not find any increase in the level of endogenous IAA in tobacco cells on transformation to the auxin-autonomous phenotype. Similarly, in habituated tissues, both Kutacek et al (1981) and Coumans-Gilles et al (1982) found no significant difference in endogenous levels of IAA between habituated and non-habituated tissues of tobacco and sugarbeet respectively.

Estimates of putative endogenous IAA levels in the Lactuca tissues analysed in this present work were found to be higher at three separate stages throughout a 21 day culture period, suggesting that an alteration in the level of endogenous IAA may occur on conversion to the habituated state. These quantifications were, however, subsequently shown to be overestimates of the levels present in the tissues due to the presence of contaminants in the extracts and therefore could only be regarded as preliminary measurements. The GC-MS estimates were thought to be closer to the actual levels present in the tissues

and these quantifications also indicated a difference in IAA levels between the two tissue types.

In contrast to these observations, Koves and Szabo (1987) found that levels of endogenous IAA were lower in a habituated tobacco tissue than in a non-habituated culture. The results of Koves and Szabo (1987) appeared to show that the lower IAA levels in the habituated culture were related to a lower growth rate in the habituated tissue. A similar situation may have been observed in the Lactuca tissues analysed in this present work, where the levels of endogenous IAA were higher in the habituated culture at all stages analysed during a 21 day culture period. The doubling time of the habituated tissue was 4 days compared to the 5 days of the non-habituated culture and the duration of the lag phase was longer in the non-habituated tissue (Fig. 3.23) which could account for the difference in the timing of maximum IAA levels measured in the two tissue types. The difference in endogenous levels of IAA measured could, in fact, be related to the growth rate of the tissue and not causally related to the auxin-independent phenotype.

The crucial question as to whether the endogenous IAA concentration in cultured plant tissues is linked to the capacity for auxin-independent growth requires further comparative analyses of endogenous IAA levels to be undertaken in auxin-dependent and independent tissues using the most advanced physicochemical techniques available.

The problems encountered in this present work in

attempting to analyse endogenous IAA levels meant that the estimates obtained could only be considered as preliminary. Further investigations of endogenous IAA levels in this tissue would require a more extensive purification procedure to adequately remove contaminant compounds prior to quantification by HPLC-F and an alternative internal standard to ^{3}(H)-IAA as this isotope was found to degrade too rapidly to be suitable for this type of analysis.

If the levels of endogenous IAA are important in the regulation of cell division in plants and in auxin-autotrophic growth, it is important to understand the mechanisms by which auxin levels are regulated in plant tissues. If the regulation of IAA metabolism is important to the expression of auxin-autonomous growth, determination of the relative importance of each of the components which control IAA metabolism to the overall regulation of endogenous IAA levels could help to identify which metabolic alterations occur on conversion to the auxin-auonomous state.

Coumans-Gilles et al (1982) measured equivalent levels of endogenous IAA in habituated and non-habituated sugarbeet tissue cultures. The habituated tissues contained a higher level of both auxin protectors and peroxidases and the authors suggested that this alteration in the rate of IAA destruction in the habituated sugarbeet tissue counteracted the build up of endogenous IAA levels in this tissue caused by enhanced biosynthesis. Atsumi (1980) also believes that the level of auxin protectors are important

in governing endogenous IAA levels in cultured plant cells and in the determination of auxin-autonomous growth, by altering the rate of IAA destruction.

IAA biosynthesis was not detected (< 0.1ng of trp converted to IAA) in 10 day old tissues of either the habituated or non-habituated Lactuca cultures. Most of the tryptophan fed to the tissues appeared to have been metabolised in other anabolic pathways, presumably protein synthesis (Butenko 1979) or may have been compartmentalised and unavailable for IAA biosynthesis (Rekoslavskaya et al 1986). This attempt to analyse IAA biosynthesis in the callus tissues was hindered by the difficulties involved in recovering metabolites of ^{14}C tryptophan from callus extracts. The metabolites involved in IAA biosynthesis are known to be very labile and are present in extremely low quantities. Great care must therefore be taken during isolation of the indole extracts in the nature of the solvents used in both the extraction procedure and TLC analysis.

The only observed difference in ^{14}C tryptophan metabolism between the habituated and non-habituated tissues was in the level of tryptophan metabolism. There was evidence to suggest that ^{14}C tryptophan had been recovered only from the non-habituated tissue, suggesting a lower level of tryptophan metabolism in this tissue. This was in fitting with the slightly lower growth rate measured in this culture.

A compound was recovered from both tissues

with an Rf of 0.41, close to that of tryptamine (0.42), suggesting that the tryptamine pathway of IAA biosynthesis could be important in this tissue. The presence of tryptamine as a ^{14}C tryptophan metabolite was not, however, confirmed in this work due to a lack of time for further analysis.

Bandurski et al (1977) have proposed that auxin conjugation could play a major role in the regulation of IAA levels in plant tissues. A growth reduction in Zea mays seedlings, caused by a growth-inhibitory light flash, was found to be accompanied by an increase in the level of free IAA and a reduction in esterified forms of IAA. The release of IAA from conjugates appeared to be related to the reduction in growth rate measured in these tissues. The removal of light from non-habituated Lactuca callus tissues caused a significant reduction in growth rate. On analysis of the IAA content of non-habituated tissues grown in both the light and dark, estimates of the levels of free IAA were seen to have been reduced in the dark-grown tissue. It must, however, be recognised that these investigations were only preliminary estimations of the levels of free IAA in the tissues as a result of the problems involved in quantification, highlighted in Chapter 3.

Attempts to estimate the levels of esterified IAA in these tissues were unsuccessful as a result of contamination in the hydrolysed extract causing problems in HPLC analysis. It could not, therefore, be established whether the observed reduction in free IAA levels was the

result of conjugation of the IAA or of other metabolic fates ie. reduced biosynthesis, increased catabolism, reduced protection of IAA or its release from the cells into the surrounding growth medium. The observation that the habituated tissues were not inhibited by culture in the dark in a similar fashion to the non- habituated culture could, however, indicate a difference in the regulation of auxin metabolism in the two tissues, if the reduction in growth rate was directly related to alterations in the level of IAA. Tissue culture offers great potential for the study of alterations in IAA concentration in response to environmental stimuli as the in vitro environment is easily manipulable and cultured plant tissues represent a largely homogeneous mass of dividing cells in which growth and endogenous IAA levels can both be easily monitored.

The concentration of endogenous auxin appears to play a crucial role in the regulation of cell division in cultured plant tissues. Dramatic fluctuations in IAA levels were monitored in both habituated (10 fold) and non-habituated (100 fold) Lactuca tissues during a 21 day culture period. Similar fluctuations in IAA levels have been observed in several other cultured plant tissues both auxin-dependent (Moloney et al 1983) and independent (Pengelly et al 1986) and these fluctuations may be correlated with specific stages of the growth cycle in cultured cells. Robinson (1982) has reported that the endogenous levels of IAA in an auxin-dependent Acer cell suspension were closely correlated to the number of cells

in mitosis. In this same tissue, Moloney et al (1983) found that a peak in IAA levels correlated with the period of intensive growth in this tissue. Similarly, high levels of endogenous IAA have been measured during the exponential growth phase of habituated and non-habituated sugarbeet cells (Coumans-Gilles et al 1982).

Leguay and Guern (1977) have reported that the level of the synthetic auxin, 2,4-D, was important in governing cell division in an Acer cell suspension. They found that endogenous 2,4-D levels were reduced at the onset of the stationary phase and suggested that a critical threshold level of endogenous auxin was required below which cell division would not occur. However, Moloney et al (1983) did not find that the endogenous levels of 2,4-D correlated directly with growth events in the Acer tissue. 2,4-D levels peaked prior to a peak in IAA levels but were very low by the onset of active cell division, whereas IAA levels were high at this critical stage. Where the levels of endogenous IAA in cultured plant tissues appear to play a role the regulation of cell division, the role of endogenous auxin concentration in auxin-autotrophic growth is less clear.

It may not, of course, be the concentration of endogenous auxin which is the determining factor in the regulation of cell division in plant tissues. Certain authors believe that the number of available binding sites for auxins and other growth regulators may be the limiting factor in plant cell growth and division (Trewavas 1982).

In relation to the problem of growth regulator autonomy, this would mean that alterations in auxin and cytokinin requirements expressed in these tissues may occur through variation in the number or type of receptors for these growth regulators present in the plant tissue.

There is evidence to suggest that auxin-binding sites can be induced by incubation with 2,4-D (Trewavas 1980, Van der Linde 1985). Van der Linde et al (1985) also demonstrated that more than one type of auxin-binding site can be induced in tobacco tissue cultures in response to the growth regulator content of the culture medium. However, despite extensive reports of specific binding sites in plant tissues for auxin and other growth regulators, there has been no clear demonstration yet, that these binding sites act as receptors in the transduction of hormonal activity. Advances in this area of plant growth regulator research will be crucial in determining whether it is tissue sensitivity or the concentration of endogenous growth regulators which is responsible for the regulation of plant cell growth and division.

Understanding the role of IAA in the regulation of cell division could be the key to finding the mechanism by which auxin-autrotrophic growth is induced and maintained in cultured plant tissues. Auxin is capable of inducing cell division in quiescent tissues (Yeoman and Mitchell 1970, Yasuda 1974) but the exact mechanism by which auxin switches on cell division is not clear. Parenti et al(1973) has reported that the dedifferentiation of N.

glauca explants was accompanied by the amplification of a portion of the DNA. Interestingly, this same satellite DNA also appears to have been found in habituated but not in non- habituated tobacco tissue cultures analysed by Durante et al (1986) and therefore may be in some way related to the initiation of cell growth and the capacity for auxin-autonomous growth.

There are also reports that specific genes are expressed in plant tissues on exposure to auxin. Several authors have reported the production of specific sets of mRNA's on response to auxin addition (Theologis 1986) and these auxin-regulated gene products could play a crucial role in the regulation of plant cell growth process. Further work in determining the nature of these auxin-regulated gene products will, undoubtedly, be valuable in identifying the mode of action of auxin in cell division (Guilfoyle and Hagen 1988).

Direct demonstrations of auxin involvement in the induction of cell division are not common due to the technical difficulties involved in isolating cell division from all of the other intracellular processes in which auxin is involved. The use of auxin antagonists to reduce the activity of endogenous IAA, by displacing bound auxin from binding sites in the tissue, is a novel approach to the problem of investigating the role of IAA in intracellular growth processes.

DNA synthesis was found to be significantly reduced in habituated tissues incubated with the auxin

antagonist, 1-napthoxyacetic acid. This reduction of DNA synthesis may have been, at least, partially the result of a reduced uptake of the nucleoside at the plasmalemma measured in the presence of the antagonist. This suggests that auxin involvement in DNA synthesis was at least, in part, indirect. It must, however, be acknowledged that the ^{3}(H) thymidine may not have been taken up solely into the DNA of the cells as it may also be incorporated into proteins and this could have affected the results obtained. A clearer demonstration of the fate of the ^{3}(H) thymidine could have been obtained by extraction of the DNA from the habituated tissue using CsCl gradient or by using autoradiography techniques to demonstrate the location of the radiolabel within the cells.

The uptake of ^{3}(H) thymidine was found to be only partially sensitive to auxin, as it could not be completely inhibited even at very high concentrations of antagonist. These results suggested that auxin was involved in the regulation of ^{3}(H) thymidine uptake at the plasmalemma.

A similar demonstration of auxin involvement in the uptake of glucose and amino acids has been reported in auxin-autonomous crown gall tissues of tobacco and potato (Rausch et al 1984). It would appear that auxin is involved in the regulation of the uptake of essential solutes at the plasmalemma and regulation of the uptake of essential precursors for growth could ultimately be the limiting step in the regulation of cell growth and division. Whether the control of the uptake of essential nutrients differed in

habituated and non-habituated tissues would be worth examining to determine whether any link exists with the capacity for auxin-autotrophic growth, as has previously been suggested by Wood and Braun (1965).

The problem of habituation in tissue culture remains a peculiar phenomenon. A clearer understanding of the mechanisms responsible for the initiation and maintenance of this condition is, however, of great importance as it will enable a wider understanding of plant growth regulation of cell division both *in vitro* and *in vivo*. Such knowledge should also enable a fuller exploitation of the potentialities of plant cells grown in culture.

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Appendix A (Figures 2.1 and 2.2):

Habituated tissue

Passage No.	Wt/Wo	ln Wt/Wo	Σ ln Wt/Wo
1	28.9	3.3	3.3
2	33.4	3.5	6.8
3	43.4	3.7	10.5
4	37.9	3.6	14.1
5	42.2	3.7	17.8
6	56.6	4.0	21.8
7	48.3	3.9	25.7
8	14.0	2.5	28.1
9	67.8	4.2	32.3
10	42.8	3.7	36.0
11	65.4	4.0	40.0
12	84.0	4.4	44.4
13	31.5	3.3	47.7
14	63.4	4.1	51.8
15	56.7	3.9	55.7
16	44.2	3.7	59.4
17	99.0	4.5	63.9
18	63.2	3.8	67.7
19	17.3	2.7	70.5
20	44.4	3.6	74.0
21	48.6	3.7	77.8

$y = -0.81 + 3.75\ x \qquad r = 0.998$

$td = \frac{0.69}{3.75} = 0.184 \times 21 = 3.9$ days.

Non-habituated tissue

Passage No.	Wt/Wo	ln Wt/Wo	Σ ln Wt/Wo
1	8.5	2.1	2.1
2	10.1	2.2	4.4
3	10.0	2.2	6.6
4	10.5	2.3	8.8
5	6.5	1.8	10.6
6	30.3	3.2	13.7
7	25.5	3.1	16.8
8	11.9	2.2	19.0
9	53.1	3.8	22.7
10	21.5	2.9	25.7
11	24.5	3.0	28.7
12	13.9	2.5	31.2
13	10.4	2.2	33.5
14	19.4	2.8	36.3
15	29.8	3.1	39.4
16	24.3	3.1	42.5
17	27.4	3.2	45.7
18	29.5	3.3	49.0
19	23.7	3.0	52.0
20	32.9	2.3	55.3
21	41.8	3.7	58.9

$y = -2.2 + 2.82\ x \qquad r = 0.998$

$td = \frac{0.69}{2.82} = 0.245 \times 21 = 5.1$ days

Each data point is the mean of 8 replicates.

Appendix B : Losses during purification (Fig. 3.2)

Integrated area of standard IAA a.u. = 1.96×10^5 (1st analysis)
1.79×10^5 (2nd analysis)

Step 7, losses from centrifugal freeze drying was incorporated into each loss determined below.

step	Area a.u. x 10^5	Area a.u. corr.	Area a.u. std.	% recovery	mean	% contribution to loss
1	1.59	1.70	1.96	87		
	1.62	1.73	1.96	88	87	8.4
	1.42	1.52	1.79	85		
2	1.62	1.73	1.79	97	97	2.0
3	1.73	1.85	1.96	94	94	3.9
4	1.12	1.20	1.96	61		
	1.29	1.38	1.96	70		
	0.92	1.03	1.79	58	64	23.2
	1.10	1.18	1.79	66		
5	1.51	1.61	1.96	82		
	1.68	1.80	1.96	92		
	1.31	1.40	1.79	79	84	10.3
	1.37	1.46	1.79	82		
6	0.48	0.52	1.96	26		
	0.53	0.57	1.96	29		
	0.36	0.39	1.79	22	26	47.7
	0.43	0.46	1.79	26		
7	1.86		1.96	95		
	1.98		1.96	101		
	1.68		1.96	94	94	7.0
	1.47		1.79	82		
				Total	155	100

Appendix C:

Standard IAA curve on ODS analytical HPLC column (Fig.3.3).

ng IAA injected	0.35	0.7	1.75	2.45	3.5
Area a.u.	2.7	7.9	17.8	26.8	33.0
	3.2	9.0	15.7	22.2	31.6
	3.1	6.9	15.5	25.6	38.3
	3.8	7.4	16.2	22.7	33.8
	3.0	6.6	17.0	22.4	32.4
	2.5	6.6	17.5	24.2	32.1
	2.7	7.6	18.4	23.6	37.0
	3.5	7.0	18.8	20.0	34.5
	1.8	7.2	21.8	/	/
	2.1	7.2	16.8	/	/
$\bar{x}$	2.8	7.4	17.6	23.4	34.1
(s.d.)	(0.6)	(0.7)	(1.8)	(2.1)	(2.4)

Appendix D:

Standard IAA curve on ODS preparative HPLC column (Fig. 3.4).

ng IAA injected	0.35	0.7	1.75	2.45	3.5
Area a.u.	5.2	8.7	23.1	29.2	45.7
	5.5	8.3	25.0	40.1	45.7
	5.1	8.6	28.2	40.5	46.6
	5.5	8.7	26.4	36.2	43.9
	5.5	9.0	24.9	32.1	47.7
$\bar{x}$	5.3	8.7	25.5	35.6	45.9
(s.d.)	(0.2)	(2.3)	(1.9)	(5.0)	(1.4)

Appendix E:

standard IAA curve on GC-MS(SIM) (Fig.3.7).

Rt (mins)	ng injected	Area of abundance 189	Area of abundance 130	Ratio 189/130
9.28	10.0	76.0	289.0	26
9.26	1.0	9.2	35.9	26
9.25	0.1	1.1	3.9	27
9.25	0.05	0.2	1.2	19

Appendix F:

(Fig. 3.8)

Plant sample		dpms/ng ^{3}(H) IAA recovered x 10^{6}	dpms/ng ^{3}(H) IAA added x 10^{6}	Radioactive purity %	^{3}(H) IAA added (ng) (X)
Habituated					
Day	7	2.6	3.3	81	5.73
	14	1.5	3.5	43	3.04
	21	2.0	3.1	63	4.46
Non-habituated					
Day	7	1.4	2.4	58	4.1
	14	1.2	3.0	39	2.8
	21	0.5	2.4	20	1.4

Appendix G:

Calibrations of standard methyl IAA on GC-MS

Methyl IAA injected (ng)	Area of abundance 130 (a.u.)	Area of abundance 189 (a.u.)	Ratio of 189/130
100	50.0	13.0	27
50	22.0	6.8	30
25	8.7	2.0	24
d_2 IAA-Me			
200	5.1	1.3	26
	132		
	36.8	12.1	33
100	320	111.0	34
50	140	51.0	36
25	67	22.0	32
10	88	27.0	31
1	14	4.2	30
0.1	2.5	0.7	28
habituated plant extract			
	37.9	12.0	31
100	33.5	9.9	30
10	8.5	2.4	28
1	0.5	0.1	25
0.1	0.2	0.09	41

Appendix H (Table 3.5):

Habituated

day			dpm/ng	mean dpm/ng	X (ng)	IAA (ng/g fwt)	mean IAA (ng/g fwt ± SEM)
		1	2558				
	1	2	2742	2800	5.73	271	
		3	589				
		1	1105				
7	2	2	961	1020	5.73	750	556 ± 145
		3	994				
		1	1370				
	3	2	995	1183	5.73	646	
		3	1184				
		1	4397				
	1	2	4169	4492	3.04	89	
		3	4909				
		1	11039				
14	2	2	11945	10618	3.04	37	67 ± 16
		3	7520				
		1	5317				
	3	2	5530	5334	3.04	75	
		3	5156				
		1	23558				
	1	2	21685	23566	4.46	23	
		3	25454				
		1	22979				
21	2	2	21880	22651	4.46	24	36 ± 13
		3	23093				
		1	9420				
	3	2	9226	9347	4.46	62	
		3	9394				

Ci = 2.68×10^5 dpms/ng

Non-habituated

day			dpm/ng	mean dpm/ng	X (ng)	IAA (ng/g fwt)	mean IAA (ng/g fwt ± SEM)
		1	73045				
	1	2	77992	75582	4.1	5.2	
		3	75708				
		1	76290				
7	2	2	77191	77190	4.1	5.1	5.1 ± 0.03
		3	78090				
		1	83746				
	3	2	77500	76932	4.1	5.1	
		3	69550				
		1	579				
	1	2	593	571	2.8	646	
		3	541				
		1	688				
14	2	2	594	698	2.8	528	531 ± 66
		3	812				
		1	891				
	3	2	767	881	2.8	418	
		3	985				
		1	20537				
	1	2	24117	23539	1.4	7.4	
		3	25964				
		1	13363				
21	2	2	12723	13191	1.4	10.2	10.3 ± 2
		3	13486				
		1	9365				
	3	2	9895	9736	1.4	13.3	
		3	9947				

Appendix I (Table 3.7):

Corrected habituated estimates

day			dpm/ng	mean dpm/ng	X (ng)	IAA (ng/g fwt)	mean IAA (ng/g fwt ± SEM)
		1	4050				
	1	2	4342	4433	5.73	171	
		3	4908				
		1	1750				
7	2	2	1479	1586	5.73	482	355 ± 94
		3	1530				
		1	2108				
	3	2	1575	1853	5.73	412	
		3	1875				
		1	6749				
	1	2	6413	6899	3.04	58	
		3	7535				
		1	16956				
14	2	2	20456	16327	3.04	23	43 ± 10
		3	11569				
		1	8165				
	3	2	8507	8197	3.04	48	
		3	7918				
		1	36224				
	1	2	33362	36255	4.46	14	
		3	39160				
		1	35352				
21	2	2	33558	34779	4.46	15	23 ± 8
		3	35426				
		1	14492				
	3	2	14194	14379	4.46	39	
		3	14452				

Corrected non-habituated estimates

day			dpm/ng	mean dpm/ng	X (ng)	IAA (ng/g fwt)	mean IAA (ng/g fwt ± SEM)
		1	157549				
	1	2	170164	164298	4.1	1.3	
		3	165182				
		1	165848				
7	2	2	166490	167366	4.1	1.2	1.3 ± 0.02
		3	169761				
		1	181450				
	3	2	168478	167041	4.1	1.2	
		3	151196				
		1	1258				
	1	2	1290	1438	2.76	256	
		3	1177				
		1	1496				
14	2	2	1290	1517	2.76	243	230 ± 20
		3	1765				
		1	1937				
	3	2	1667	1915	2.76	192	
		3	2140				
		1	44645				
	1	2	52428	53053	1.42	4.7	
		3	62087				
		1	29155				
21	2	2	27713	28748	1.42	6.0	6.4 ± 1
		3	29376				
		1	20360				
	3	2	21477	21153	1.42	8.5	
		3	21623				

Appendix J (Figs. 4.4 and 4.5):

Growth of habituated and non-habituated tissues in the light and dark.

	Weeks in culture	Wt/Wo	ln Wt/Wo	$\mathcal{E}$ ln Wt/Wo
Habituated				
Light	3	68.6	4.22	8.5
	6	74.33	4.3	
Dark	3	111.6	4.7	9.5
	6	124.0	4.8	

Light : $y = -0.002 + 1.41\ x$

Dark : $y = -0.03 + 1.57\ x$

Non-habituated				
Light	3	134.7	4.9	9.13
	6	69	4.2	
Dark	3	20.45	3.02	6.99
	6	52.9	3.97	

Light : $y = 0.12 + 1.51\ x$

Dark : $y = -0.16 + 1.13\ x$

Appendix K:

(Figure 5.4)

Control

Time (mins)	cpm/20mg	$\bar{x}$	s.d.	% SEM	cpm/mg
0	44				
	64	54	14.1	18	2.7
20	427				
	505	466	55.2	8.5	23.2
40	666				
	730	698	45.3	4.6	34.9
80	769				
	823	796	38.2	3.5	39.8
120	974				
	1242	1108	189.5	12.0	55.4
160	2238				
	2618	2428	268.7	7.8	121.4

+1-NOA

0	113				
	81	97	22.6	16.3	4.9
5	67				
	53	60	9.9	16.5	3.0
10	128				
	156	142	19.8	9.2	7.1
20	196				
	252	224	39.6	12.7	11.2
40	290				
	266	278	17.0	4.2	13.9
80	314				
	274	294	28.3	6.8	14.7
120	372				
	480	426	76.4	12.7	21.3
160	581				
	683	632	72.1	7.8	31.6

(Figure 5.5)

Control

Time (mins)	$\times 10^2$ cpm/20mg	$\bar{x}$	s.d.	% SEM	$\times 10^2$ cpm/mg
0	11.7 14.4	13	1.9	15	0.65
20	64 46	55	12.7	23	2.8
40	63 87	75.0	17.0	12.0	3.7
80	132 180	155.8	33.9	15.6	7.8
120	194 251	222.4	40.3	6.4	11.1
160	227 348	288	85.6	21.0	14.4

+1-NOA

0	/	4	/	/	0.1
20	25 34	29.4	6.2	14.9	1.5
40	47 66	56.6	13.4	16.8	2.8
80	106 130	118.0	17.0	10.2	5.9
120	112 136	123.8	16.7	9.5	6.2
160	178 211	194.6	23.3	8.5	9.7

(Figure 5.6)

Control

Time (mins)	$\times 10^2$ cpm/20mg	$\bar{x}$	s.d.	% SEM	$\times 10^2$ cpm/mg
0	10				
	7	8.1	2.2	19.1	0.4
20	64				
	76	70.0	8.1	8.1	3.5
40	92				
	73	82.6	13.6	11.3	4.1
80	214				
	242	228	19.8	14.0	11.4
120	238				
	294	266	39.6	14.8	13.3
160	337				
	376	356.6	27.9	5.5	17.8

+1-NOA, +1-NAA

0	10				
	12	11.0	2.0	12.7	0.6
20	46				
	58	51.6	8.5	11.6	2.6
40	48				
	54	50.8	15.7	14.5	2.5
80	108				
	88	98.0	14.1	10.2	4.9
120	136				
	177	156.4	29.0	13.1	7.8
160	176				
	197	186.6	14.8	5.7	9.3

Appendix L (Fig. 5.3):

Compound		Control	IAA	PCIB	2-NAA	1-NOA
Replicate	1	519	530	498	385	343
	2	422	518	551	436	246
	3	546	605	397	398	267
$\bar{x}$		496	551	482	406	285
(+/- s.d.)		65	47	78	27	51
% of control		100	111	97	82	57
% SEM		7.6	4.9	9.3	3.8	10.3

Appendix M (Figs 5.7-5.12) :

Test compound	cpms/20mg sample Control	25	50	100	200	400conc.(μM)
1-NOA	44352	41212	20136	16098	12249	17964
	42480	38817	16982	14455	17687	15214
	53527					
$\bar{x}$	46786	40014	18559	15277	14968	16589
s.d.	5912	1694	2230	1162	3845	1945
% basis	100	86	40	3	32	35
% SEM	7.3	3.0	8.5	5.4	18	8.3

Test compound	cpms/20mg sample Control	25	50	100	200	400conc.(μM)
2-NAA	32541	22100	21197	14333	14885	15539
	34505	32557	17738	18669	13826	14966
	39015					
$\bar{x}$	35354	27328	19468	16501	14355	15253
% basis	100	77	55	47	41	43

Test compound	cpms/20mg sample					
	Control	25	50	100	200	400conc.(μM)
1-NAA	50622	44965	22054	11650	18726	12107
	67887	56194	32312	30367	20233	11341
	44097	38199	36548	35912	18343	13569
	75373	36634	16426	13116	19500	12670
$\bar{x}$	59495	43998	26835	22761	19201	12422
s.d.	14587	8898	9229	12210	840	939
% basis	100	74	45	38	32	21
% SEM	12.2	10.0	17.2	27	2.2	3.8

Test compound	cpms/20mg sample					
	Control	25	50	75	100	200
IAA (1)	16787	15260	11755	15130	5047	7988
	6448	11583	4794	6110	7943	7531
	8236					
$\bar{x}$	10490	13422	8275	10620	6495	7760
s.d.	5526	2600	4922	6378	2048	323
% basis	100	128	79	101	62	74
% SEM	30	13.7	42	42	22	3
IAA (2)	29987	20623	21307	25884	28514	13871
	30696	20865	18626	29654	29489	16614
	24461					
$\bar{x}$	28381	20744	19967	27770	29001	15243
s.d.	3414	171	1896	2666	689	1940
% basis	100	73	70	98	102	54
% SEM	7	0.6	6.7	6.8	1.7	9
JOINT %	100	101	75	100	82	64

Test compound	Control	25	50	100	200	400conc.(μM)
Benzoic acid (1)	35038	33152	26290	20730	15222	20266
	16828	29546	18271	12746	22411	11426
	13363	25211	16418	14404	19358	11938
	17929	21124	30051	16839	13691	9306
	25383					
$\bar{x}$	21707	27258	22758	16180	17678	13234
s.d	8645	5221	6481	3468	3976	4824
% basis	100	125	105	75	81	61
% SEM	17.8	9.6	14.0	10.7	11.2	18.2
Benzoic acid (2)	18316		17212	28702	22253	14075
	22937		17105	31344	21158	12050
	20226					
$\bar{x}$	20493		17159	30023	21706	13063
s.d.	2322		75.6	1868	774	1432
% basis	100		84	147	106	64
% SEM	6.5		0.3	44	2.5	7.8
JOINT %	100	125	95	111	94	63

Test compound	Control	25	50	100	200	400conc.(μM)
Phenoxyacetic acid	23962	29533	27006	28563	34173	20096
	29945	35295	20166	27048	21237	27006
	27420	20036	20788	17847	20698	16547
	22585	36681	15362	21976	22708	
	34728					
$\bar{x}$	27728	30386	20830	23858	24704	21216
s.d.	4864	7563	4778	4899	6370	5319
% basis	100	110	75	86	89	77
% SEM	7.8	12.5	11.5	10.3	12.9	14.5